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Bioactive aristolactams from *Piper umbellatum*

Turibio Kuiate Tabopda [a,b,*], Joseph Ngoupayo [b], Jiawei Liu [a], Anne-Claire Mitaine-Offer [d], Sheraz A. Khan Tanoli [c], Shamsun Nahar Khan [c], Muhammad Shaiq Ali [c], Bonaventure Tchaleu Ngadjui [b], Etienne Tsamo [b], Marie-Aleth Lacaille-Dubois [d], Bang Luu [a]

[a] *Laboratoire de Chimie Organique des Substances Naturelles, LC3-UMR 7177 CNRS, Université Louis Pasteur, Centre de Neurochimie, 5 Rue Blaise Pascal, 67084 Strasbourg, France*
[b] *Département de Chimie Organique, Université de Yaoundé I, BP 812 Yaoundé, Cameroon*
[c] *H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Pakistan*
[d] *Université de Bourgogne, Faculté de Pharmacie, Laboratoire de Pharmacognosie, UMIB UPRES EA 3660, 7, Bd Jeanne d'Arc, B.P. 87900, 21079 Dijon Cedex, France*

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ABSTRACT

Four alkaloids named piperumbellactams A–D (**1**–**4**) were isolated from branches of *Piper umbellatum* together with known *N*-hydroxyaristolam II (**5**), *N*-*p*-coumaroyl tyramine (**6**), 4-nerolidylcatechol (**7**), *N*-*trans*-feruloyltyramine, *E*-3-(3,4-dihydroxyphenyl)-*N*-2-[4-hydroxyphenylethyl]-2-propenamide, β-amyrin, friedelin, apigenin 8-C-neohesperidoside, acacetin 6-C-β-D-glucopyranoside, β-sitosterol, its 3-O-β-D-glucopyranoside and its 3-O-β-D-[6′-dodecanoyl]-glucopyranoside. Glycosidase inhibition, antioxidant and antifungal activities of these compounds were evaluated. Compounds **1**–**3** showed moderate α-glucosidase enzyme inhibition with IC_{50} values 98.07 ± 0.44, 43.80 ± 0.56 and 29.64 ± 0.46, respectively. In DPPH radical scavenging assay, compounds **2**, **3** and **6** showed potent inhibitory activity while compounds **4**, **5** and **7** showed potent antifungal activity.

1. Introduction

Piper is a large genus of herbs or somewhat woody climbers found in the warm, humid regions of world. *Piper umbellatum* is widely used in Cameroon traditional medicine for the treatment of poisoning, pitting oedema, foetal malpresentation, filariasis, rheumatism, haemorrhoids, dysmenorrhea and general pains. A variety of compounds have been isolated from *Piper* species including amides, lignans, neolignans, hydroquinones, alkaloids, terpenes, oxygenated cyclohexane and benzoic acid derivatives (Parmar et al., 1997; Wu et al., 1997; Yamaguchi et al., 2006). Many aristolactams have been isolated from *Piper* species (Olsen et al., 1993; Singh et al., 1996; Desai et al., 1988, 1990; Ruangrungsi et al., 1992) but only few with N-OH (Wan et al., 2007), N-OCH_3 (Lin et al., 2006; Kumar et al., 2003), *N*-glucosyl or *N*-acetyl function (Kumar et al., 2003). Some of these compounds are reported to be active anti-platelet activating factor (PAF) (Shen and Hussaini, 1990), phospholipase A_2 (PLA_2) inhibitory (Núñez et al., 2005), anti-inflammatory (Lin et al., 2006), antioxidant (Yamaguchi et al., 2006) and cytotoxic (Sun et al., 1987; Duh et al., 1990; Ruangrungsi et al., 1992) principles. There is no thorough phytochemical investigation on *P. umbellatum* available to date. However, Núñez et al. (2005) reported the inhibitory effects of *P. umbellatum* extracts and 4-nerolidylcatechol towards myotoxic PLA_2 from Bothrops snake venoms and the isolation of 2-(4′,8′-dimethylnona-3′,7′-dienyl)-8-hydroxy-2-methyl-2H-chromene-6-carboxylic acid methyl ester. The present study reports the isolation and characterization of four new and three known active alkaloids together with nine other secondary metabolites from methanol extract of *P. umbellatum*. Additionally, α-glucosidase enzyme inhibition, the ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) and antifungal activity were analysed, as well.

Glucosidases are involved in the synthesis of glycoproteins and the lysosomal catabolism of glycoconjugates. Alpha-glucosidase EC 3.2.1.20 is a group of enzymes whose specificity is directed mainly towards the exohydrolysis of 1,4-α-glucosidic linkages, and that hydrolyze oligosaccharides rapidly, relative to polysaccharides, which are hydrolyzed relatively slowly, or not at all. The intestinal enzyme also hydrolyzes polysaccharides, catalyzing the reactions of EC 3.2.1.3, and, more slowly, hydrolyzes 1,6-α-D-glucose links. There are many α-glucosidases with many functions in the mammal. The involvement of sucrase, isomaltase and maltase in digestion is just one of these functions. Alpha-glucosidase inhibitors are widely used in the treatment of patients with type 2 diabetes. They delay the absorption of carbohydrates from the small intestine and thus have a lowering effect on postprandial blood glucose and

* Corresponding author. Address: Département de Chimie Organique, Université de Yaoundé I, BP 812 Yaoundé, Cameroon. Tel.: +237 7976633; fax: +237 2221873.
E-mail address: ttabopda@yahoo.fr (T.K. Tabopda).

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insulin levels (Sonei et al., 2000). They are potentially useful as antiviral, antimetastatic and immunomodulatory agents. They are also potentially active against HIV-1 infection (Asano et al., 1997). Nojirimycin (Inouye et al., 1968; Reese and Parrish, 1968) and acarbose (Truscheit et al., 1981) are known to be powerful α-glucosidase inhibitors derived from microorganisms. In the search for alternative α-glucosidase inhibitors, we speculated that analysis of the secondary metabolites synthesized by plants would be a promising line of inquiry.

Antioxidants are substances which can prevent, stop or reduce oxidation damage (Ramarathnam et al., 1995). They appear to minimize the number of oxidative DNA mutations and proteins modifications by scavenging harmful reactive oxygen species (ROS) (Diplock et al., 1998). Therefore, they are able to protect the human body from several diseases (Alzheimer's disease, rheumatoid arthritis, cataracts, diabetes, Parkinson's disease, AIDS) attributed to the reactions of radicals (Nizamuddin, 1987; Takao et al., 1994). Use of synthetic antioxidants to prevent free radical damage has been reported to involve toxic side effects (Cornwell et al., 1998; Faure et al., 1990; Feher and Pronai, 1993), making attractive the search for antioxidant and scavenger natural compounds.

The clinical relevance of fungal diseases increases enormously due to the increasing of the immunocompromised host in the second half of the 20th century including infected with HIV, transplant recipients and patients with cancer (Clark and Hajjeh, 2002; Hage et al., 2002). The crude mortality from opportunistic fungal infections still exceeds 50% in most human studies and has been reported to be as high as 95% in bone marrow transplant recipients infected with *Aspergillus* sp. (Romani, 2004). The commonly used antifungals, such amphotericin-B, miconazole, ketoconazole, fluconazole and clotrimazole are toxic or limited in their spectrum and efficiency (Wakabayashi et al., 1998; Helmerhorst et al., 1999). For these reasons, there is a need for new active molecules, particularly those from plant extracts, which can serve as lead for further development in antifungal chemotherapy.

2. Results and discussion

Branches of *P. umbellatum* were extracted at room temperature with methanol. Purification of the extracts produced four new alkaloids named piperumbellactams A–D (**1–4**) along with known *N*-hydroxyaristolam II (**5**) (Wan et al., 2007), *N*-*p*-coumaroyl tyramine (**6**) (Lin et al., 2006), 4-nerolidylcatechol (**7**) (Kijjoa et al., 1980) and other compounds identified as *N*-*trans*-feruloyltyramine (Singh et al., 1996; Chang et al., 2000), *E*-3-(3,4-dihydroxyphenyl)-*N*-2-[4-hydroxyphenylethyl]-2-propenamide (Lajide et al., 1995), β-amyrin (Domínguez and Alcorn, 1985), friedelin (Parmar et al., 1997), apigenin 8-C-neohesperidoside (Rayyan et al., 2005), acacetin 6-C-β-D-glucopyranoside (Bylka and Matławska, 1997), β-sitosterol (Domínguez and Alcorn, 1985), its 3-O-β-D-glucopyranoside (Parmar et al., 1997) and its 3-O-β-D-[6′-dodecanoyl]-glucopyranoside (Woldemichael et al., 2003).

Compound (**1**) was isolated as an amorphous powder. The HRESIMS mass spectrum established the molecular formula $C_{17}H_{13}NO_4$. Its UV (MeOH) spectrum was characteristic of an aristolactam sharing absorptions at 196, 209, 231, 242, 274, 283, 311 and 379 nm (Wu et al., 1997). Its IR (KBr) spectrum showed absorption bands at 3361, 1700 and 1639 cm^{-1} suggesting the presence of hydroxyl and lactam carbonyl groups. The ^{13}C NMR spectrum (Table 1) showed signals at δ 168.9, 60.4 and 57.4 which were consistent with the presence of a lactam and two methoxy groups (see Fig. 1).

The ^1H NMR (DMSO-d_6, 300 MHz) spectrum of compound (**1**) showed six aromatic protons, a broad D_2O-exchangeable hydroxyl proton at δ 10.86 and two aromatic methoxy groups at δ 4.06 (3H, *s*) and 4.04 (3H, *s*), respectively. The signals at δ 9.13 (1H, *dd*, 6, 3) assigned to H-5, and the signals at δ 7.58 (2H, *m*) and 7.96 (1H, *dd*, 6, 3) assigned to the remaining protons of ring C suggested four adjacent aromatic protons at C-5 (δ 126.0), C-6 (δ 127.3), C-7 (δ 128.0) and C-8 (δ 129.6), respectively. The HMBC correlations between δ 7.88 (*s*) and C-1 (δ 122.0), C-3 (δ 154.7), C-4 (δ 150.9), C-11 (δ 123.8), C-12 (δ 168.9) and between δ 7.15 (*s*) and C-5a (δ 126.4), C-8 (δ 129.6), C-8a (δ 135.3), C-11 (δ 123.8) and C-10 (δ 135.6) indicated that the two other aromatic protons were assigned to H-2 and H-9, respectively. This is in great agreement with the aristolactam skeleton. The singlet at δ 10.86 (1H, *s*) was attributed to an exchangeable proton belonging to *N*-hydroxyl group. This proton showed long range correlation with the signal at δ_C 135.6 (C-10), 123.8 (C-11), and 122.0 (C-1). The two methoxy groups at δ 4.06 (s) and 4. 04 (s) were unequivocally assigned at C-3 and C-4 according to the HMBC and ROESY correlations (Desai et al., 1988). The HMBC correlations between the aromatic protons at δ 7.58 (H-6, H-7) and C-5 (δ 126.0), C-5a (δ 126.4), C-8 (δ 129.6) and C-8a (δ 135.3), between the aromatic proton at δ 9.13 (H-5) and C-4a (δ 120.4) and C-7 (δ 128.0), and between the aromatic proton at δ 7.93 (H-8) and C-5a (δ 126.4), C-6 (δ 127.3) and C-9

Table 1
^1H and ^{13}C NMR spectroscopic data of **1–4** in DMSO-d_6, d in ppm, J in Hz

Position	**1**		**2**		**3**		**4**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	–	122.0	–	114.9	–	114.7	–	121.9
2	7.88 (*s*)	110.4	7.77 (*s*)	109.0	7.70 (*s*)	107.4	7.92 (*s*)	108.0
3	–	154.7	–	149.9	–	148.2	–	144.7
4	–	150.9	–	148.7	–	150.2	–	147.0
4a	–	120.4	–	124.8	–	124.8	–	120.3
5	9.13 (*dd*, 6, 3)	126.0	9.27 (*dd*, 6, 3)	125.4	9.27 (*dd*, 6, 3)	125.0	9.11 (*dd*, 6, 3)	125.7
5a	–	126.4	–	127.2	–	126.3	–	125.7
6	7.58 (*m*)	127.3	7.54 (*m*)	127.1	7.51 (*m*)	127.4	7.59 (*m*)	127.1
7	7.58 (*m*)	128.0	7.54 (*m*)	127.9	7.51 (*m*)	127.8	7.59 (*m*)	127.9
8	7.96 (*dd*, 6, 3)	129.6	7.93 (*dd*, 6, 3)	129.2	7.81 (*dd*, 6, 3)	128.4	7.90 (*dd*, 6, 3)	129.3
8a	–	135.3	–	134.6	–	134.2	–	134.7
9	7.15 (*s*)	105.1	7.13 (*s*)	104.9	7.09 (*s*)	105.8	7.13 (*s*)	106.2
10	–	135.6	–	135.6	–	134.4	–	134.8
11	–	123.8	–	116.3	–	115.5	–	122.3
12	–	168.9	–	169.4	–	170.9	–	168.9
OCH_2O	–	–	–	–	–	–	6.28 (*s*)	102.4
3-OCH_3	4.06 (*s*)	57.4	4.05 (*s*)	57.6	–	–	–	–
4-OCH_3	4.04 (*s*)	60.4	–	–	–	–	–	–
N-OH	10.86 (*s*)	–	10.66 (*s*)	–	–	–	–	–
N-OCH_3	–	–	–	–	4.07	63.7	4.08	63.4

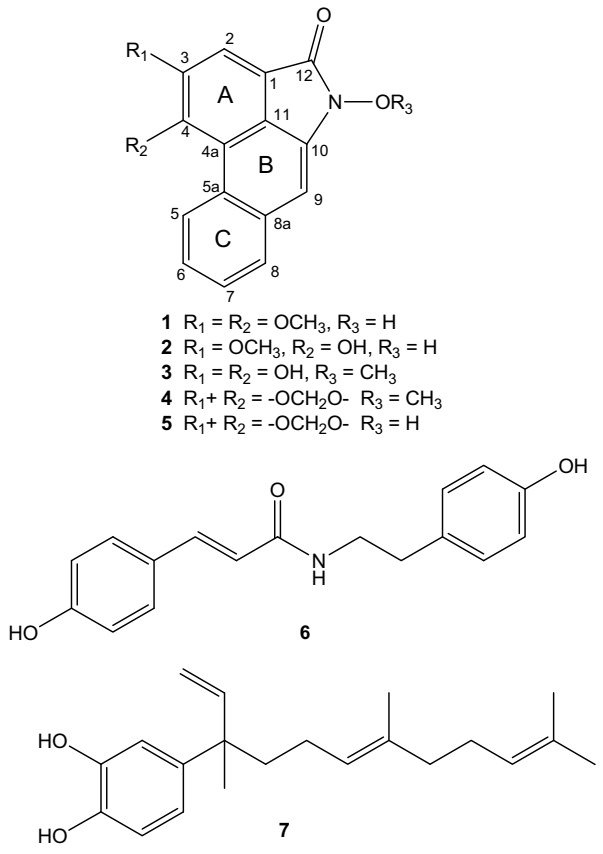

Fig. 1. Structures of isolated compounds.

(δ 105.1) confirmed the connectivities of the complete structure of compound **1**. Significant correlations between H-5 (δ 9.13) and H-6 (δ 7.58), H-7 (δ 7.58) and H-8 (δ 7.96) were observed in the ^{1}H–^{1}H ROESY spectrum (Fig. 2). Thus, taking into consideration the above data and analysis, the structure of compound (**1**) was elucidated as 10-amino-3,4-dimethoxy-*N*-hydroxyphenanthrene-1-carboxylic acid lactam, named piperumbellactam A.

Piperumbellactam B (**2**), 10-amino-4-hydroxy-3-methoxy-*N*-hydroxyphenanthrene-1-carboxylic acid lactam was isolated as an amorphous powder. The HRESIMS mass spectrum established the molecular formula $C_{16}H_{11}NO_4$. Its UV, IR and NMR spectra were similar to those of piperumbellactam A except that the ^{1}H and ^{13}C NMR spectrum of compound (**2**) (Table 1) showed one methoxy group instead of two as in compound (**1**). The ^{1}H NMR spectrum of compound (**2**) showed a broad D_2O-exchangeable signal at δ 10.66 (1H, *s*) assigned to N-OH on the basis of the HMBC correlations between δ_H 10.66 (s) with C-10 (δ_C 135.6), C-11 (δ_C 116.3) and C-1 (δ_C 114.9). On the basis of all these data, the methoxy group was proposed as residing at C-3 or C-4. The cross peak in the HMBC experiment between δ 4.05 (3H, *s*, 3-OCH_3) and δ 149.9 (C-3), and the correlation in the ROESY experiment between the signal at δ 7.77 (1H, *s*, H-2) and the methoxy group (δ 57.4) confirmed the linkage of the methoxy group at C-3 (δ 154.7). This deduction was supported by the literature according to which, due to a downfield effect of two *ortho* substituents, hindered aromatic methoxy signal appeared at δ_C 59–63 (Chaudhuri et al., 1978). In the HMBC spectrum, the aromatic proton at δ7.77 (H-2) showed five cross-peaks to four sp^2 carbon signals at δ_C 114.9 (C-1), 149.9 (C-3), 148.7 (C-4) and 116.3 (C-11) and to a lactam carbonyl at δ_C 169.4 (C-12). The HMBC correlations observed from H-6 (δ 7.54) and H-7 (δ 7.54) to C-5, C-5a, C-8 and C-8a, from H-5 (δ 9.27) to C-4a and C-7, from H-8 (δ 7.93) to C-5a, C-6 and C-9 and H-9 (δ 7.13) to C-5a, C-8, C-10, C-11 completed the aristolactam structure assignment. The ^{1}H–^{1}H COSY spectrum indicated correlations between H-5 (δ 9.27) and H-6 (δ 7.54) and between H-8 (δ 7.93) and H-7 (δ 7.54) indicating the presence of one set of four mutually coupled protons.

Piperumbellactam C (**3**) was isolated as an amorphous powder. The HRESIMS mass spectrum established the molecular formula $C_{16}H_{11}NO_4$. The UV, IR and ^{13}C NMR spectrum of compound (**3**) was similar to those of piperumbellactam A and B and when NaO-Ac and H_3BO_3 were added, the UV spectrum of **3** showed a bathochromic shift, indicating the presence of a *ortho*-dihydroxyl group at C-3 and C-4 (Mabry et al., 1970). The ^{1}H NMR do not display signal of *N*-hydroxyl group at δ 10.66 suggesting that N atom may be substituted by a methoxyl group instead of hydroxyl group as in compounds (**1**) and (**2**). Furthermore, in the HMBC experiment, no correlation was observed between methoxy protons (δ_H 4.07, *s*) and oxygenated aromatic carbons at δ 148.2 and 150.2. The ^{13}C NMR spectrum (Table 1) exhibited signal of N-OCH_3 at δ_C 63.7 together with signals of two phenolic hydroxyl at δ_C 148.2 (C-3) and 150.2 (C-4). In the ROESY spectrum, a correlation was observed between H-9 (δ 7.09) and the H_3 of the methoxyl group. Detailed analyses of the ^{13}C NMR, ^{1}H NMR, COSY, HSQC, ROESY and HMBC data led to conclusion that piperumbellactam C (**3**) had the same skeleton as **1** and **2**. Therefore, the structure of piperumbellactam C (**3**) was deduced as 10-amino-3,4-dihydroxy-*N*-methoxyphenanthrene-1-carboxylic acid lactam.

Piperumbellactam D (**4**), was isolated as an amorphous powder. The HRESIMS mass spectrum established the molecular formula $C_{17}H_{12}NO_4$. Its UV (MeOH) spectrum was characteristic of an aristolactam. Its IR (KBr) spectrum exhibited bands at 1700 (lactam carbonyl group) and 924 (methylenedioxyphenyl group) cm^{-1}. The ^{13}C NMR spectrum (Table 1) showed signals at δ_C 168.9, 102.4 and 63.4 which were consistent with the presence of a lactam carbonyl, a methylenedioxyphenyl and a methoxy groups. The ^{1}H NMR spectrum of compound (**4**) (Table 1) showed six aromatic protons, a methoxy group (δ 4.08, *s*) and a methylenedioxyphenyl group by the signal at δ 6.28 (2H, *s*). The signals at δ_H 9.11 (1H, *dd*, 6, 3), 7.59 (2H, *m*) and 7.92 (1H, *dd*, 6, 3) suggested four adjacent aromatic protons at C-5, C-6, C-7 and C-8, respectively. The HMBC correlations of H-2 to C-1, C-3, C-4, C-11, C-12 and H-9 to C-5a, C-8, C-8a, C-11 and C-10 indicated that the two other aromatic protons, assigned for H-2 and H-9, respectively, appeared as singlet at δ_H 7.92 (1H, *s*) and 7.13 (1H, *s*). This is in great agreement with the aristolactam skeleton as suggested that the methylenedioxyphenyl group is located at C-3 and C-4. ROESY-correlations (Fig. 2) between OCH_3 and H-9 confirm the spatial proximity of these protons. Analysis of ^{1}H and ^{13}C NMR spectral data (Table 1) showed chemical shifts similar to those of piperumbellactam C (**3**), but illustrated the presence of methylenedioxyphenyl. Hence, a singlet at δ 6.28 assignable to a methylenedioxy group fused to the C-3 and C-4 positions of ring A could be proposed, as this proton showed long-range ^{1}H–^{13}C correlations with C-3 (δ 144.7) and C-4 (δ 147.0) in the HMBC spectrum. Based on the above, the structure of piperumbellactam D (**4**) was deduced as 10-amino-3,4-methylenedioxyphenyl-*N*-methoxyphenanthrene-1-carboxylic acid lactam.

The antioxidant activity of compounds **1**–**3** and **6** was evaluated. At the concentration of 10 μM (Table 2), these compounds were able to scavenge the DPPH radical with 13.1%, 67.8%, 86.4% and 61.8%, respectively. Compounds (**2**), (**3**) and (**6**) exhibited the most potent radical scavenging activity with IC_{50} 17.4, 8.1 and 13.7 μM, respectively. The greatest effectiveness of compound (**3**) than the others was possibly due to the presence of *ortho*-dihydroxy group which upon donating hydrogen radicals will give higher stability to their radical forms (Shahidi and Wandasundara, 1992).

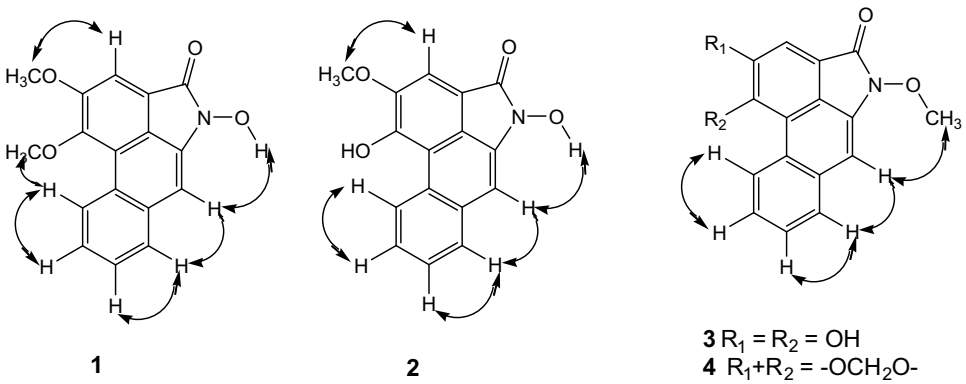

Fig. 2. Key ROESY correlations of **1**, **2** and **3**.

Table 2
Radical scavenging activity of compounds **1**, **2**, **3** and **6** (at 10 μM)

Compounds	% Scavenging of DPPH
1	13.1 ± 0.7
2	67.8 ± 0.5
3	86.4 ± 0.9
6	61.8 ± 0.4
Caffeic acid	58.0 ± 0.6

Compounds **1**–**3** were also evaluated for their possible glycosidase enzyme inhibitory activity again α-glucosidase, β-glucosidase and α-mannosidase. These compounds showed weak to moderate and selective α-glucosidase inhibition with IC_{50} 98.07, 43.80 and 29.64 μM, respectively. The glycosidase inhibition of compounds **1**–**3** are shown in Table 3.

Compounds **4**, **5** and **7** were screened for their in vitro antifungal activity against *Trichophyton longifusus*, *Aspergillus flavus*, *Microsporum canis*, *Fusarium solani*, *Candida albicans* and *C. glabrata*. The results of this study (Table 4) showed compounds (**4**) and (**5**) to be more antifungal against more species as compared to extracts and 4-nerolidylcatechol (**7**). The miconazole and amphotericin B were chosen as standard antifungal drugs in the assay. Furthermore, compounds (**4**) and (**5**) show higher antifungal activity than amphotericin B against *A. flavus* and *T. longifusus*. Compounds **4** and **5** were slightly more potent probably due to the ability of the methylenic carbon in methylenedioxyphenyl (MDP) to form stable carbene under oxidation. The reported compounds are good candidates as antifungal agents.

3. Experimental section

3.1. General

^1H NMR, ^{13}C NMR and two-dimensional COSY, ROESY, HSQC and HMBC experiments were performed on a Bruker Avance DPX instrument (300 and 75 MHz). The 2.50 and 40.0 ppm resonances of residual CD_3SOCD_3 were used as internal references for ^1H and ^{13}C NMR spectra, respectively. Analytical and preparative TLC were carried out on pre-coated silica gel 60 F_{254} plates (Merck, 0.25 and 0.50 mm thickness, respectively). The plates were checked under UV light (254 nm) and developed with vanillin and H_2SO_4 in EtOH (Analytical TLC). Mass spectra were recorded on a micro TOF instrument. IR spectra were obtained with a Nicolet AVATAR 320 FT-IR spectrophotometer. UV spectra were recorded on a Bio-TeK spectrophotometer. All melting points were determined on a micro-melting point apparatus and are uncorrected.

3.2. Plant material

Branches of *P. umbellatum* were collected from Bandjoun, West province of Cameroon, in may 2006. Plant was identified by Dr. Pierre Nana, Botanist, at the National Herbarium, Yaounde, Cameroon, and a voucher specimen No. 6516/SRF/CAM was deposited.

3.3. Extraction and isolation

The air-dried powdered branches of *P. umbellatum* (1 kg) were extracted with MeOH (5 L × 3) at room temperature. The solvent was evaporated to dryness giving a MeOH extract (40 g) which was partitioned successively between H_2O and Et_2O (500 mL × 3), followed by $CHCl_3$ (1 L × 3) and *n*-BuOH (1 L × 3). The $CHCl_3$ fraction (20 g) was subjected to column chromatography (CC) on silica gel (40–63 μm, 9 × 73 cm) eluted with a gradient of EtOAc/*n*-hexane and MeOH/AcOEt, and 25 fractions (1–25, each 250 mL) were collected. Fractions 10–15 (950 mg) were combined and separated on a silica gel column (25–40 μm, 3 × 45 cm) eluted with AcOEt/*n*-hexane, 1:1 to yield β-sitosterol (430 mg), sitosterol-3-O-β-D-glucopyranoside (128 mg) and piperumbellactam A (**1**, 7 mg). Fractions 17–18 (320 mg) were rechromatographed on a silica gel column (40–63 μm, 5 × 25 cm) using MeOH/AcOEt 10:90 as eluent to give two main fractions A (54 mg) and B (143 mg). Fraction A was subjected to preparative normal phase TLC and piperumbellactam C (**3**, MeOH/AcOEt 95:5, Rf = 0.41, 10 mg) was obtained. Fraction B was chromatographed on

Table 3
Glycosidase inhibitory activity of compounds **1**–**3**

Compound	α-D-Glucopyranoside (yeast) IC_{50} ± s.e.m. (μM)	β-D-Glucopyranoside (sweet almonds) IC_{50} ± s.e.m. (μM)	β-D-Mannopyranoside (jack bean) IC_{50} ± s.e.m. (μM)
Piperumbellactam A (**1**)	98.07 ± 0.44	218.33 ± 0.13	NI
Piperumbellactam B (**2**)	43.80 ± 0.56	322.14 ± 0.96	NI
Piperumbellactam C (**3**)	29.64 ± 0.46	113.29 ± 0.56	752.39 ± 0.77
1-Deoxynojirimycin	426 ± 8.14	–	–

NI: No inhibition at 800 μM concentration.

Table 4
In vitro antifungal activity of extracts, fractions and compounds from *P. umbellatum*

Name of fungus	Inhibition of samples								Std. drug MIC μg/mL	% Inhibition of std. drug/mm
	Mext	Hex	Chl	But	F4	**4**	**5**	**7**		
Trichophyton longifusus	40	13	62	11	64	73	89	50	Miconazole	70
Candida albicans	55	21	73	–	88	101	108	55	Miconazole	110.8
Aspergillus favus	22	–	15	–	27	35	51	10	Amphotericin B	20
Microsporum canis	62	33	71	22	84	90	87	50	Miconazole	98.4
Fusarium solani	37	08	51	–	55	63	65	49	Miconazole	73.25
Candida glabrata	71	46	83	51	91	101	99	78	Miconazole	110.8

Values are % inhibition of radial growth. Mext, methanol extract; Hex, hexane fraction; Chl, chloroforme fraction; But, *n*-butanol fraction; F4, fraction 4. Concentration of sample = 200 μg/mL of DMSO. Incubation temperature (period) = 28 ± 1 °C (7 days). (–) = No activity.

Sephadex LH-20 eluted with MeOH to afford piperumbellactam B (**2**, 12 mg) and *N*-*p*-coumaroyl tyramine (**6**, 4 mg). Another methanol extract (31 g) was obtained in the same conditions as described above and macerated with *n*-hexane (400 mL × 3) to give the *n*-hexane fraction. The insoluble residue (28 g) was suspended in water and partitioned between $CHCl_3$ (400 mL × 3; 4.7 g) and *n*-BuOH (400 mL × 3; 17.5 g). All the fractions were subjected to antifungal test. The chloroform fraction, which showed appreciable antifungal activity, was then concentrated to a brown viscous mass under reduced pressure and subjected to vacuum liquid chromatography (VLC) on silica gel (80 g) using a gradient of hexane–EtOAc (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 0:10; each 300 mL, v/v) as eluent to yield nine fractions (F1–F9). Fraction F4, *n*-hexane–AcOEt (6:4, v/v) in which activity was localized, was further subjected to repeated CC by using $CHCl_3$–MeOH–H_2O (40:10:1) as eluent. 4-Nerolidylcatechol (**7**) was obtained together with a mixture of two compounds which was then separated using preparative thin layer chromatography (PTLC) (three times), eluted with $CHCl_3$/MeOH/H_2O (40:10:1) to yield (**5**) (27 mg, Rf 0.38) and (**4**) (39 mg, Rf 0.44).

3.4. Enzyme inhibition assay

Glycosidase inhibition assay was performed according to the slightly modified method of Matsu et al. (1996). α-D-Glucosidase (E.C.3.2.1.20), β-D-glucosidase (3.2.1.21) and α-D-mannosidase (3.2.1.24), were purchased from Wako Pure Chemical Industries Ltd. (Wako 076-02841). The enzyme inhibition was measured spectrophotometrically at 37 °C for 30 min using 0.7 mM *p*-nitrophenyl α-D-glucopyranoside (PNP-G), and *p*-nitrophenyl β-D-glucopyranoside as a substrate at pH 6.9, then at pH 4.0 using 1 mM *p*-nitrophenyl α-D-mannopyranoside and 500 units/mL enzymes, in 50 mM sodium phosphate buffer containing 100 mM NaCl. 1-deoxynojirimycin (0.425 mM) was used as positive control. The increment in absorption at 400 nm due to the hydrolysis of PNP-G by glycosidase was monitored on microplate spectrophotometer (Spectra Max, Molecular Devices USA). The absorbance of the reaction mixture was recorded at 400 nm at 1 min intervals in a temperature-controlled chamber at 37 °C. The linear reaction velocity (change in absorbance per minute) was calculated from the gradient of the linear portion of the reaction profile and used to determine the α-glucosidase activity.

3.5. Antioxidant assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity was measured according to the procedure described by Christov et al. (2005). The compounds were dissolved in ethanol (0.21 mg/mL). The solutions analyzed (250 μL) were diluted to 2 mL with ethanol and 1 mL of 0.02% DPPH/ethanol solution was added. The resulting mixture was thoroughly mixed and the absorbance measured at 517 nm after 30 min. The scavenging activity was determined by comparison of the absorbance with blank (100%), containing only DPPH and solvent. Caffeic acid has been chosen as positive control.

3.6. Antifungal assay

In vitro antifungal Bioassay was performed using agar tube dilution method (Ohtani et al., 1991; Kusumi, 1993; Brass et al., 1979). Each compound (1.5 mg) dissolved in 1 mL of sterile dimethylsulphoxide (DMSO) served as stock solution. Sabouraud dextrose agar (SDA) (4 mL) was added into screwcaped tubes and autoclaved at 121 °C for 15 min and then cooled to 50 °C. The non-solidified SDA media was poisoned with 66.6 μL of the stock solution to give 200 μg compound/mL of SDA. Tubes were then allowed to solidify in slanting position at room temperature. Each tube was inoculated with 4 mm diameter piece of the inoculum removed from a 7 days old culture of fungi. For non-mycelial growth, an agar surface streak was employed. Inhibition of fungal growth was observed after 7 days of incubation at 28 ± 1 °C. Negative and positive control experiments were also carried out with DMSO and reference antifungal drugs, respectively.

3.7. Piperumbellactam A (*1*)

Amorphous powder; ^1H (300 MHz, DMSO-d_6) and ^{13}C (75 MHz, DMSO-d_6) NMR: see Table 1; UV λ_{max} (MeOH) nm: 196, 209, 231, 242, 274, 283, 311, 379; IR ν_{max}^{KBr} cm^{-1}: 3261, 1700, 1609, 1604, 1483, 1305; HRESIMS: *m*/*z* 295.2689 $[M]^+$ (calcd. for $C_{17}H_{13}NO_4$, 295.2697).

3.8. Piperumbellactam B (*2*)

Amorphous powder; ^1H (300 MHz, DMSO-d_6) and ^{13}C (75 MHz, DMSO-d_6) NMR: see Table 1; UV λ_{max} (MeOH) nm: 194, 203, 226, 247, 274, 280, 316, 378; IR ν_{max}^{KBr} cm^{-1}: 3315, 1701, 1607, 1601, 1478; HRESIMS: *m*/*z* 281.2876 $[M]^+$ (calcd. for $C_{16}H_{11}NO_4$, 281.2863).

3.9. Piperumbellactam C (*3*)

Amorphous powder; ^1H (300 MHz, DMSO-d_6) and ^{13}C (75 MHz, DMSO-d_6) NMR: see Table 1; UV λ_{max} (MeOH) nm: 203, 226, 246, 279, 313, 378; UV λ_{max} (MeOH + NaOAc + H_3BO_3) nm: 204, 229, 247, 285, 315, 389; IR ν_{max}^{KBr} cm^{-1}: 3317, 1700, 1604, 1483; HRESIMS: *m*/*z* 281.2867 $[M]^+$ (calcd. for $C_{16}H_{11}NO_4$, 281.2863).

3.10. Piperumbellactam D (*4*)

Amorphous powder; ^1H (300 MHz, DMSO-d_6) and ^{13}C (75 MHz, DMSO-d_6) NMR: see Table 1; UV λ_{max} (MeOH) nm: 197, 209, 231,

243, 276, 282, 311, 380; IR ν_{max}^{KBr} cm^{-1}: 3293, 1705, 1615, 1601, 1473, 1321, 924; HRESIMS: *m/z* 294.2611 $[M+H]^+$ (calcd. for $C_{17}H_{12}NO_4$, 294.2623).

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