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Antimicrobial and cytotoxic constituents from native Cameroonian medicinal plant *Hypericum riparium* ☆

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ABSTRACT

Bioassay guided fractionation of *Hypericum riparium* leaves extract has resulted in the isolation and characterization of three new compounds namely chipericumin E (**1**), hyperenone C (**3**), and hyperixanthone (**5**), together with twenty known compounds. Their structures were elucidated based on comprehensive interpretation of spectroscopic and spectrometric data. Compounds **1**–**4**, and **6**–**8** displayed moderate antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and cytotoxic effects on the human gastric cell line BGC-823 with IC_{50} values ranging from 6.54 to 18.50 μM.

1. Introduction

Plants of the family Hypericaceae comprise ca. 9 genera and 560 species that are widely distributed in temperate regions of the world. *Hypericum* species are used in folk medicine and credited with a long list of medicinal uses, including antiviral, antifungal and antitumor; and for the treatment of neurological disorders and depression [1,2]. Their analgesic and sedative properties have also been demonstrated. These ethnobotanical uses prompted many scientists to focus their research on the phytochemical and pharmacological investigations of the genus *Hypericum*. As results, several metabolites of diverse classes with interesting biological properties were isolated and characterized. Example included phenylpropanoids, flavonoids, proanthocyanidines, xanthones, benzophenones, phloroglucinols and naphtodianthrones [2–7]. Therefore, it has been noted that interest in the chemical study of *Hypericum* spp. is due not only to their uses in medicine, but also to the development of chemosystematic investigations. As part of our continuing search for new bioactive metabolites from plants belonging to the genus *Hypericum*, we carried out the chemical and biological investigations on the leaves of *Hypericum riparium*, a small tree of 5 m high widely dispersed over Central, Eastern and South Africa. In Cameroon, this plant is used to treat epilepsy, mental diseases, gastric disorders, and microbial infections such as ringworm and scabies [8,9]. Previously, cytotoxic and antimicrobial xanthones,

☆ Dedicated with best wishes to Prof. Dr. Hartmut Laatsch on the occasion of his 69th birthday.

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phloroglucinols, coumarins and triterpenes have been reported from the roots and stem bark of *H. riparium* [8,9].

Bioassay guided fractionation and purification of the dichloromethane-methanol (1:1) extract from *H. riparium* leaves resulted in the isolation and identification of three new compounds namely chipericumin E (**1**), hyperenone C (**3**), and hyperixanthone (**5**), together with twenty known compounds. We herein report the isolation and structural elucidation of the new compounds (**1**, **3** and **5**), as well as the full characterization (^{1}H and ^{13}C NMR) of 1,5-dihydroxy-2-methoxyxynthone (**6**). Evaluation of the cytotoxic and antibacterial activities of the crude extract and isolated compounds is also reported.

2. Materials and methods

2.1. General experimental procedures

IR spectra were recorded on a Bio-Rad-FTS-135 spectrometer (KBr pellets; in cm^{-1}). UV spectra were taken with a Shimadzu UV2401PC, while the CD spectra were measured on an Applied Photophysics. Optical rotations were recorded on a Jasco P-1020 polarimeter. NMR spectra were acquired with Bruker-DRX-600 and Bruker-DRX-500 instruments with $SiMe_4$ as an internal standard in room temperature (δ in ppm and *J* in Hz). ESI-MS and ESI-HR-MS were recorded on an *API-Qstar-Pulsar* Bruker instrument. Silica gel (SiO_2; 100–200 or 200–300 mesh; Qingdao Marine Chemical Ltd. Co., PR China), silica gel *H* (60 mm; Qingdao Marine Chemical Ltd. Co., PR China), Sephadex LH-20 (Pharmacia) and MCI gel CHP-20P (75–150 mm; Mitsubishi Chemical Co.) were used for column chromatography (CC). Preparative TLC was performed on silica gel GF254 (Qingdao Marine Chemical Ltd. Co., PR China). Preparative HPLC was conducted using an Agilent Technologies 1100 series with an Agilent Technologies 1100 series MWD detector and a SunFire™ prep C18 OBD™ (250 × 19 mm, 5μm). TLC was carried out on glass precoated silica gel GF254 plates (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, PR China).

2.2. Extraction and isolation

The fine and powdered leaves of *H. riparium* (2.4 kg) were extracted by maceration at room temperature with a mixture of CH_2Cl_2–MeOH (1:1) (3 × 4 l) for 72 h. Evaporation under reduced pressure of the filtrate afforded a 75.7 g of crude extract. A portion (70 g) of the crude extract was subjected to column chromatography on silica gel using a stepwise of $CHCl_3$–MeOH gradient; forty fractions were collected and combined on the basis of their TLC profiles into 5 major fractions: *FrA* (6.63 g, $CHCl_3$–MeOH 100:0), *FrB* (10.50 g, $CHCl_3$–MeOH 100:5 → 100:10), *FrC* (9.42 g, $CHCl_3$–MeOH 100:10), and *FrD* (20.43 g, $CHCl_3$–MeOH 100:20 → 100:30). Preliminary biological assays have been carried out in all the five fractions. Hexadecanol crystallized overnight in *FrA* and was filtered of and washed with MeOH. The filtrate was rechromatographed over a silica gel column (Φ 30 mm × *L* 500 mm) using EtO_2–Me_2CO (100:0 → 100:3) to afford compound **4** (20 mg) and an oily residue. *FrB*, which contained chlorophyll, was firstly subjected to chromatography over MCI gel (MeOH–H_2O 70:30 → 100:0 follow by Me_2CO). From this chromatography, the collected fractions without chlorophyll were combined and further separated through a silica gel column (Φ 30 mm × *L* 500 mm) using a gradient of EtO_2–Me_2CO (100:0 → 100:10) to furnish six sub-fractions (*FrB-1* to *FrB-6*). Compound **3** (2 mg) was obtained from the purification of *FrB-1* over a Sephadex LH-20 ($CHCl_3$–MeOH 1:1). *FrB-2* was purified over a silica gel column using EtO_2–Me_2CO (100:1) as the isocratic system to afford compounds **10** (12 mg) and **15** (3 mg). In the same conditions, the separation of *FrB-3* provided compounds **14** (4 mg) and **7** (2 mg). *FrB-5* was also chromatographed over silica gel using EtO_2–Me_2CO (100:3) as the isocratic system to afford compounds **1** (1.3 mg) and **20** (6 mg). *FrB-6* was applied on a Sephadex LH-20 column using $CHCl_3$–MeOH (1:1) to give compounds **11** (5 mg) and **18** (2 mg). *FrB-4* was purified by HPLC using acetonitrile–H_2O (70:30) to produce compounds **8** (7 mg) and **6** (5 mg). *FrC* (10 g), also containing chlorophyll was applied through an MCI gel column. From this fractionation, three main sub-fractions were obtained. *FrC-1* was separated by prep-TLC (EtO_2–Me_2CO 80:20) to yield compounds **9** (3 mg) and **12** (3 mg). Compound **16** (80 mg) crystallized from *FrC-3* and was filtered of and washed with $CHCl_3$. The filtrate was further chromatographed over a silica gel column using a stepwise gradient of $CHCl_3$–Me_2CO. From this chromatography, compounds **19** (6 mg) and **21** (100 mg) precipitated in *FrC-3b*. Purification of *FrC-3a* by HPLC (ACN–H_2O 70:30) gave compounds **2** (2.7 mg), **5** (2 mg) and **13** (13 mg). *FrD* was subjected to a silica gel column (Φ 40 mm × *L* 500 mm) using $CHCl_3$–Me_2CO–MeOH (100:2:0 → 100:10:5) to yield **17** (11 mg) and **22** (16 mg).

Chipericumin E (1): Colorless oil, $[\alpha]^{20}_D = +11.99^o$ (*c* 0.50, MeOH); UV (MeOH) λ_{max}: 240 (ε 8615), 283 (9538); CD (MeOH) λ_{ext}: 220 (Δε +16.1), 246 (+10.1), 271 (+9.9), 294 (−2.2), 305 (+1.8); IR (KBr) ν_{max}: 3431, 2933, 1755, 1654, 1552, 1459, 1361, 1117, 1057; ^{1}H- and ^{13}C-NMR data (600 and 150 MHz, $CDCl_3$) Table 1; positive ESI-HR-MS *m/z*: 447.2677 ($[M + H]^+$; $C_{26}H_{39}O_6$, calc. 447.2668).

Hyperenone D (3): Colorless oil; $[\alpha]^{20}_D = -20.00^o$ (*c* 1.00, MeOH); UV (MeOH) λ_{max}: 203 (ε 38345). IR (KBr) ν_{max}: 2974, 1771, 1656, 1585, 1449, 1158, 1238, 763. ^{1}H- and ^{13}C-NMR data (600 and 150 MHz, $CDCl_3$) Table 2; EI-HR-MS *m/z*: 258.1125 ($[M]^+$; $C_{16}H_{18}O_3$, calc. 258.1256).

Hyperixanthone (5): Yellow amorphous solid; UV (MeOH) λ_{max}: 205 (ε 7585), 244 (12589), 318 (3162). ^{1}H- and ^{13}C-NMR data (600 and 150 MHz, DMSO-d_6) Table 3; EI-HR-MS *m/z*: 258.0543 ($[M]^+$; $C_{16}H_{18}O_3$, calc. 258.0528).

1,2-Dihydroxy-4-methoxyxanthone (6): Yellow amorphous solid; ^{1}H- and ^{13}C-NMR data (600 and 150 MHz, $CDCl_3$) Table 3. EI-HR-MS *m/z*: 258.0543 ($[M]^+$; $C_{16}H_{18}O_3$, calc. 258.0528).

2.3. Biological activities

In vitro anticancer activity. The cytotoxicity of extract, fractions and purified metabolites was evaluated on the human gastric cancer cell line (BGC-823) by sulforhodamine B (SRB) assay as described previously [10]. Briefly, the cells were cultured in RPMI 1640 medium (Sigma). Aliquots of 90 ml were seeded in 96-well flat-bottomed microtiter plates for 24 h and then treated with serial dilutions of samples (extracts, fractions and pure compounds, respectively) with the maximum concentration of 100 μg/ml for crude extract and fractions, and 20 μg/ml for pure compounds. Each sample was

Table 1
^{1}H and ^{13}C NMR data (600 and 150 MHz, $CDCl_3$) of **1**, δ in ppm, J in Hz.

Position	δ_C	δ_H	HMBC (H → C)
1	199.8	–	
2	112.1	–	
3	196.6	–	
4	66.8	–	
5	207.7	–	
6	56.6	–	
7	23.4	1.95 *m*	C-3, C-4, C-5
		1.85 *m*	
8	48.0	1.65 *m*	
9	79.5	–	
10	39.7	1.83 *m*	
11	21.9	1.82 *m*	
		1.36 *m*	
12	52.3	1.80 *m*	
13	73.7	–	
14	51.3	1.50 (*d*, 12.0)	C-4, C-13
		2.41 *m*	C-4, C-12, C-13, C-15
15	20.0	1.09 *s*	C-13, C-14,
16	27.0	1.44 *s*	C-8, C-9, C-10
17	40.6	2.70 *m*	C-1, C-6, C-19, C-18, C-22
		2.41 *m*	C-5, C-6, C-19, C-18
18	117.2	4.57 (br*t*, 6.5)	C-20, C-21
19	137.6	–	
20	17.7	1.42 *s*	C-18, C-19, C-21
21	26.1	1.51 *s*	C-19, C-20
22	23.0	1.50 *s*	C-6, C-17
23	205.1	–	
24	34.4	3.39 *m*	C-23, C-26
25	17.7	1.12 (*d*, 6.7)	C-23, C-24, C-26
26	21.7	1.25 (*d*, 6.7)	C-23, C-24, C-25

initially dissolved in DMSO and further diluted with the medium to produce different concentrations. After incubation at 37 °C in 5% of CO_2 for 48 h, cells were fixed with 25 ml of ice-cold (50% CCl_3COOH) and incubated at 4 °C for another 1 h. The plates were washed, air-dried, and stained for 15 min with 100 ml of a 0.4% SRB solution in 1% AcOH. Excessive dye was removed with 1% AcOH and the plates were then air-dried again. After drying the plates, the stain was solubilized in unbuffered Tris base (10 mM; Serva) for 30 min on a plate shaker in the dark. The absorbance was measured using a BioTek EL808 microplate reader (Molecular Devices, SPECTRA MAX 340) at a wavelength of 560 nm with a reference wavelength of 520 nm. All tests were performed in triplicate, and results expressed as IC_{50} value.

Table 2
^{1}H and ^{13}C NMR data (600 and 150 MHz, $CDCl_3$) of **3**.

Position	δ_C	δ_H
1	–	–
2	167.0	–
3	146.8	–
4	119.7	6.30 *s*
5	87.4	–
6	–	–
1′	140.5	1.95 *m*
		1.85 *m*
2′/6′	125.3	7.38 *m*
3′/5′	128.9	7.36 *m*
4′	128.2	7.30 *m*
1″	40.0	2.69 *m*, 2.76 *m*
2″	116.5	5.97*m*
3″	137.2	–
4″	26.1	1.64 *s*
5″	18.2	1.51 *s*
OMe	58.3	3.77 *s*

Table 3
^{1}H and ^{13}C NMR data (600 and 150 MHz) of **5** and **6**.

Compound	5[a]		6[b]	
Position	δ_H	δ_C	δ_H	δ_C
1	–	151.1	–	149.7
2	6.94 (*d*, 9.0)	105.4	–	142.2
3	7.30 (*d*, 9.0)	117.2	7.56 (*d*, 9.6)	121.8
4	–	148.0	7.08 (*d*, 9.6)	106.1
4a	–	144.7	–	149.1
5a	–	148.8	–	145.6
5	–	143.2	–	146.5
6	7.33 (*dd*, 1.8, 8.4)	120.8	7.34 (*dd*, 1.2, 7.8)	121.0
7	7.25 (*t*, 8.4)	124.2	7.28 (*t*, 7.8)	124.1
8	7.78 (*dd*, 1.8, 8.4)	120.4	7.59 (*dd*, 1.2, 7.8)	120.3
8a	–	114.5	–	114.7
9	–	183.1	–	182.2
9a		109.3		108.4
1–OH	12.84 *s*	–	13.10 *s*	–
OMe	3.94 (*s*)	57.2	3.85 (*s*)	56.7

[a] In DMSO-d_6.
[b] In $CDCl_3$.

Antimicrobial assay. Extract, fractions and pure compounds were evaluated for their antibacterial activities against the methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro* by using the turbidimetric method [10]. *S. aureus* was inoculated in media Müller-Hinton broth (Oxiod, CM0405, Hampshire, UK) to McFarland 0.5 and diluted with the media to a density of 1×10^6 CFU/ml. Aliquots of 90 ml were filled in 96-well U-bottomed microplate, and then treated with serial dilutions of each sample with the maximum concentration of 20 mg/ml. After being cultured at 37 °C for 24 h, the absorbance was measured at 620 nm with the microplate reader mentioned above. All tests were performed in triplicate, and results are expressed as IC_{50} value.

3. Results and discussion

3.1. Structure elucidation

Compound **1** was isolated as colorless oil. Its ESI-HR-MS exhibited a pseudomolecular ion $[M + H]^+$ at *m/z* 447.2677 (calc. 447.2668), consistent with the molecular formula $C_{26}H_{38}O_6$ (eight double bond equivalents). Its UV spectrum had absorption band at 240 and 283 nm, while the IR absorption band at 1755 cm^{-1} suggested the presence of an enolized β-triketonic system [11,12]. Analysis of its ^{1}H and ^{13}C NMR data (Table 1) revealed the presence of one isoprenyl group [δ_H 4.57 (1H, br*t*, $J = 6.5$ Hz), 2.70 and 2.41 (each 1H, *m*), 1.51 and 1.42 (each 3H, *s*)], one isopropyl group [δ_H 3.39 (1H, *m*), 1.25 (3H, *d*, $J = 6.7$ Hz), and 1.12 (3H, $J = 6.7$ Hz)], two methine groups at δ_H 1.65 (m, H-8) and 1.80 (m, H-12), three methyl groups at δ_H 1.09 (s, H_3-15), 1.44 (s, H_3-16) and 1.50 (s, H_3-22), a set of methylene groups at δ_H 1.65–2.41, two exchangeable protons (δ_H 5.41 and 6.37, respectively), two sp^3 oxygenated quaternary carbons at δ_C 79.5 (C-9) and 73.7 (C-13), one ketone group at δ_C 207.7 (C-5), one conjugated ketone at δ_C 196.6 (C-3) and one enol group at δ_C 199.8 (C-1). All these data obtained for **1** and the literature survey suggested its tricyclic nature, most likely an acylphloroglucinol [11–13].

Several *Hypericum* species have been shown to produce acylphloroglucinol derivatives possessing an acylfilicinic acid-type ring with the acyl moiety located at C-2 [13]. Careful interpretation of the ^{1}H-^{1}H COSY NMR data obtained for **1** revealed the presence of three isolated spin systems, which were C-24–C-26, C-17–C-21, and C-7–C-14. In the HMBC spectrum, correlations from H-24 (δ_H 3.39), H_3-25 (δ_H 1.12), and H_3-26 (δ_H 1.25) to the carbonyl group C-23 (δ_C 205.1), together with the correlation from H-24 to the quaternary carbon C-2 led to the connection of the methylpropanoyl group at C-2. Moreover, the prenyl group was located at C-6, while the monoterpene moiety (C-4–C-16) was established by cross-peak between H_2-14 (δ_H 1.50 and 2.41) and H_2-7 (δ_H 1.85 and 1.95) to C-4 (δ_C 66.8), C-8 (δ_C 48.0), C-11(δ_C 21.9) and C-13 (δ_C 73.7), H_2-11 (δ_H 1.36 and 1.82) to C-9 (δ_C 79.5), C-10 (δ_C 39.7), and C-12 (δ_C 52.3); H_3-15 (δ_H 1.09) to C-13, C-14 and C-12; H_3-16 (δ_H 1.44) to C-8, C-9, and C-10. Furthermore, HMBC correlations from the methyl proton H_3-22 (δ_H 1.50) to the ketone carbonyl at δ_C 207.7 (C-5) and the quaternary carbon at δ_C 56.6 (C-6), supported its location at C-6. In the light of above data, the planar structure of **1** was elucidated as shown in Fig. 1.

The relative configuration of **1** was assigned on the basis of NOESY data (Fig. 2). NOESY correlations of H-18/Me-15, H-18/H-7β, H-18/H-14β, and Me-15/H-14β and of H-8/H-7β, H-8/Me-16, and H-12/H-7α (Fig. 2) strongly supported that **1** had same relative configuration as its analogue chipericumin D [11]. The CD data obtained for **1** showed positive Cotton effects at λ_{max} 220, 271, and 305 nm and a negative Cotton effect at λ_{max} 294 nm (Fig. 1, SI) comparable to those of chipericumins C and D [11]. Thus, absolute configuration of **1** was elucidated and this compound was trivially named chipericumin E.

Compound **3** was isolated as an optically active colorless oil $[\alpha]_D = -20.00^{o}$ (*c* 1.00, MeOH). Its molecular formula $C_{16}H_{18}O_3$ (eight double bond equivalents) was assigned by the EI-HR-MS which gave a molecular ion $[M]^+$ at *m/z* 258.1125 (calc. 258.1256). The IR spectrum showed characteristic absorption bands of lactone carbonyl group at 1771 cm^{-1}, and mono-substituted aromatic ring at 1656 and 1585 cm^{-1} [13,14]. The ^{1}H NMR spectrum exhibited typical signal resonances of one mono-substituted benzene ring [δ_H 7.38 (2H, *m*), 7.36 (2H, *m*), and 7.30 (1H, *m*)], one isoprenyl group [δ_H 5.94 (1H, *m*), 2.69, 2.76 (each 1H, *m*), 1.64 and 1.51 (each 3H, *s*)], one methoxy group at δ_H 3.77 (3H, *s*) and one isolated olefinic proton at δ_H 6.30 (1H, *s*). Inspection of the ^{13}C NMR spectrum (Table 2) revealed sixteen carbon atoms, attributed to five quaternary carbons (including one ester carbonyl at δ_C 167.0, one oxygenated quatenary sp^2 carbon at δ_C 146.8 and one oxygenated quatenary sp^3 carbon at δ_C 87.4), seven methines, one methylene, two methyls and one methoxy

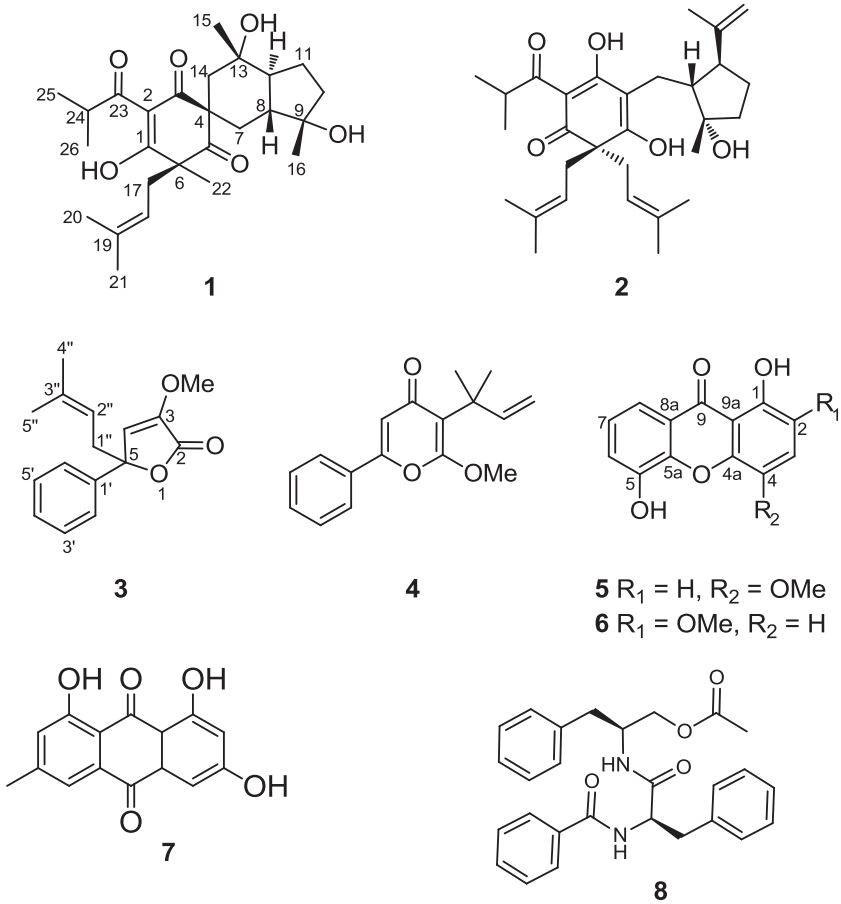

Fig. 1. Structures of compounds **1**–**8**.

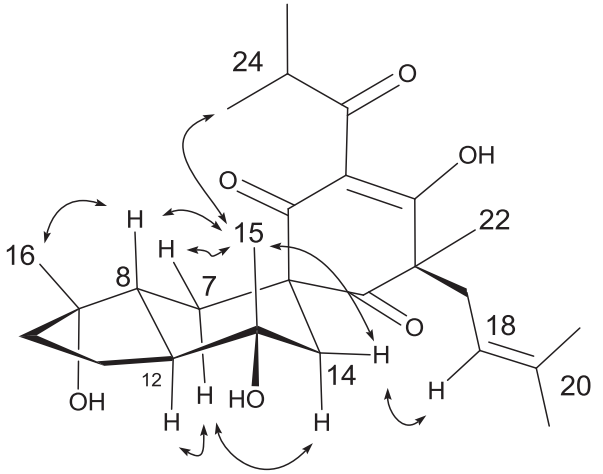

Fig. 2. Key ROE correlations of **1**.

group. The presence of the 2(5*H*)-furanone ring in the molecule was deduced from the number of DBE and by the HMBC data. The presence of one aromatic ring accounted for four while one carbonyl and one double bond from the isoprenyl group accounted for another two, which made a total of six double bond equivalents. Therefore, compound **3** was suggested to possess an additional five-membered ring with one oxygen and one double bond. This assumption was supported by the HMBC correlation from the isolated olefinic proton H-4 (δ_H 6.30) to C-2 (δ_C 167.0), C-3 (δ_C 146.8), and C-5 (δ_C 87.4) (Fig. 3). The above data showed that **3** is a prenylated butenolide bearing a phenyl group and a methoxy group. A such compound namely, 4-methoxy-3-(3-methyl-but-2-enyl)-5-phenyl-2(5*H*)-furanone with same mass and molecular formula was previously isolated from *Hypericum mysorense* [14]. But when comparing the ^{13}C NMR data of **3** to those of this structurally related compound, significant variations in the chemical shifts were observed for the furanone ring, suggesting a position change of the substituents on the furanone ring. For compound **3**, the C-5 position of the phenyl and the isoprenyl groups on this ring was determined by the analysis of the HMBC spectrum in which important correlations from H-2′ (δ_H 7.38), H-2″ (δ_H 5.97), H-1″ (δ_H 2.76) and H-4 (δ_H 6.30) to C-5 (δ_C 87.4) clearly appeared (Fig. 3). Moreover, the HMBC correlations from H-4 and the protons of the methoxy group (δ_H 3.77) to C-3 (δ_C 146.8), confirmed the location of the methoxy group at C-3. Thus, **3** was characterized as 3-methoxy-5-(3-methyl-but-2-enyl)-5-phenyl-2(5*H*)-furanone.

Compound **5** was obtained as a yellow amorphous solid. Its molecular formula, $C_{14}H_{10}O_5$ (with ten double bond equivalents) was deduced from the molecular ion peak $[M]^+$ at *m/z* 258.0543 (calc. 258.0528) in the EI-HR-MS. The UV absorption bands at 205, 244, and 318 nm suggested that **5** is a xanthone derivative [15]. The ^{1}H NMR spectrum of **5** (Table 3) revealed the presence of one hydrogen-bonded hydroxy proton at δ_H 12.84 (*s*, 1-OH) and a methoxy group at δ_H 3.94 (3H, *s*, 4-OCH_3). This spectrum also exhibited signals of an AB splitting pattern at δ_H 6.94 (1H, *d*, $J = 9.0$, H-2) and 7.30 (1H, *d*, $J = 9.0$, H-3), and of an ABC-spin system at δ_H 7.25 (1H, *t*, $J = 8.4$ Hz, H-7), 7.33 (1H, *dd*, $J = 1.8$ and 8.4 Hz, H-6), and 7.78 (1H, *dd*, $J = 1.8$ and 8.4 Hz, H-8), typical to those of 1,5-dihydroxyxanthone [16,17]. The ^{13}C NMR (Table 3) spectrum revealed characteristic signals of one ketone carbonyl group at δ_C 183.1 (C-9), one methoxy group at δ_C 57.2, and five protonated carbons at δ_C 105.4 (C-2), 117.2 (C-3), 120.4 (C-8), 120.8 (C-6), and 124.2 (C-7) and four quaternary carbon atoms of which two were oxygenated (δ_C 144.7 and 148.8). In the HMBC spectrum, the aromatic proton H-2 (δ_H 6.94) showed correlations with the oxygenated aromatic carbons C-1 (δ_C 151.1), C-4 (δ_C 148.0) and C-9a (δ_C 109.3), while H-8 correlated with C-9, C-5a, and C-8a. Furthermore, the methoxy group was located at C-4, while free hydroxy group was located at C-5, respectively (Fig. 3). From these observations, **5** was characterized as 1,5-dihydroxy-4-methoxyxanthone. Previously, this compound was reported in a review [18] as constituent of *Vismia guaramirangae*, but neither name nor structure was found in the reference cited by the authors [18]. To the best of our knowledge it has never been characterized so far. Therefore, compound **5** is reported here for the first time and trivially named hyperixanthone.

Compound **6** was also obtained as a yellow solid. Its molecular formula, $C_{14}H_{10}O_5$ determined on the basis of EI-HR-MS, suggested **6** to be an isomer of **5**. This assumption was supported by the ^{1}H NMR spectrum which showed many similarities to those of **5**. This included an AB splitting pattern at δ_H 7.08 (1H, *d*, $J = 9.6$ Hz) and 7.56 (1H, *d*, $J = 9.6$ Hz), and an ABC-spin system at δ_H 7.28 (1H, *t*, $J = 7.8$ Hz), 7.34 (1H, *dd*, $J =$ 1.2 and 7.8 Hz), and 7.59 (1H, *dd*, $J = 1.2$ and 7.8 Hz). In the ^{13}C NMR spectrum of **6**, significant variations in the chemical shifts were observed for ring A wherein C-1 and C-4 were shielded to resonate at δ_C 149.7 ($\Delta\delta_C$ −2.6 ppm) and 106.1 ($\Delta\delta_C$ −41.9 ppm), while C-2 and C-3 were deshielded to resonate at δc 142.2 ($\Delta\delta_C$ 36.8 ppm) and 121.8 ($\Delta\delta_C$ 4.6 ppm) as

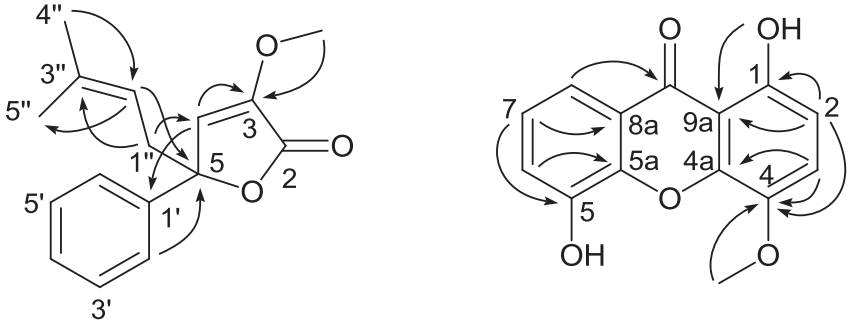

Fig. 3. Selected HMBC correlations for compounds **3** and **5**.

compared to **5**, suggesting a change on ring A. Moreover, the HMBC experiment of **6** showed pertinent correlations from the hydrogen-bonded hydroxy proton at δ_H 13.10 (*s*, 1-OH) and the protons of the methoxy group at δ_H 3.85 (3H, *s*) to an oxygenated aromatic carbon at δ_C 142.2 (C-2). Based on these observations it was evident that the ring A of compound **6** has a methoxyl group (H_3O–C-2) ortho to the chelated hydroxy group (HO–C-1). Therefore **6** was identified as 1,5-dihydroxy-2-methoxyxanthone. It was previously isolated from *H. roeperanum* [16], but only described from ^{1}H NMR data. Thus the full NMR assignments based on the extensive 1D and 2D NMR experiments are reported here for the first time.

The other structures were identified to: hypercalin C (**2**) [8], hyperenone A (**4**) [13], emodin (**7**) [19] aurentiamide acetate (**8**) [20], 3-hydroxy-5-methoxyxanthone (**9**) [8], 5-hydroxy-3-methoxyxanthone (**10**) [21], 3-hydroxy-2,4-dimethoxyxanthone (**11**) [22], 4-hydroxy-1,3-dimethoxyxanthone (**12**) [23], 2-hydroxy-3-methoxyxanthone (**13**) [24], 1,7-dihydroxyxanthone (**12**) [25], benzoic acid (**14**) [26], quercetin (**15**) [27], quercetin 3-*O*-α-L-rhamnopyranoside (**16**) [27], friedelin (**18**) [28], pachysonol (**19**) [28], betulinic acid (**20**) [28], β-sitosterol (**21**), sitosterol 3-*O*-β-D-glucopyranoside (**22**) [8], and hexadecanol.

3.2. Cytotoxic and antibacterial assays

The crude extract, fractions, as well as compounds **1**–**19** were evaluated for their cytotoxic and antibacterial activities against the human gastric cancer cell line BGC-823, and the Gram-positive bacteria *S. aureus* (Table 4). First of all, preliminary biological assessments were carried out to select samples that were able to reduce the proliferation of these two microbes up to 50% at a concentration of 20 μg/ml. Therefore, the crude extract, fractions A, B, and C, as well as compounds **1**–**4**, and **6**–**8** were selected for their dose response study. Results summarized in Table 4 showed that the crude extract, fractions A, and B displayed both cytotoxic (9.10 μg/ml ≤ IC_{50} ≤ 36.05 μg/ml) and antibacterial (2.71 μg/ml ≤ IC_{50} ≤ 48.05 μg/ml) activities. Compounds **4** and **6** exhibited moderate cytotoxicity with the IC_{50} values of 17.64 and 18.50 μM, respectively. Moreover, compounds **1–4**, **7**, and **8** showed significant antimicrobial potential (6.54 μg/ml ≤ IC_{50} ≤ 16.90 μg/ml) on *S. aureus* with aurentiamide acetate (**8**) being the most active (Table 4).

Table 4
Biological activities of extract, fractions and compounds from *H. riparium*[a].

Compounds/Fractions	Cytotoxicity IC_{50} (μM)	Antibacterial IC_{50} (μM)
	BGC–823	*S. aureus*
1	–	10.10
2	–	12.20
3	–	16.90
4	17.64	10.91
6	18.50	–
7	–	11.71
8	–	6.54
CrEx	9.74	48.05
FrA	12.61	76.58
FrB	14.01	2.71
FrC	36.05	–
FrD	–	–
Taxol	0.01	–
Ampicillin	–	0.16

[a] IC_{50} values are the means of three independent assays.

Many metabolites found reported so far from *Hypericum* spp. such as xanthones, anthraquinones, phloroglucinols, flavonoids, triterpenoids and phenyl derivatives, have been claimed to possess cytotoxic and antimicrobial activities [2,8,17,29,30]. Concerning the bioactive compounds isolated from the leave extract of *H. riparium*, the effects of hyperenone A (**4**), emodin (**7**) and aurentiamide acetate (**8**) on *S. aureus* were previously studied and the results recorded as visual minimal inhibitory concentrations (MICs) [30–32]. Our results are not so far with those found in the literature and confirm the antibacterial potential of these compounds against *S. aureus*. To the best of our knowledge, this is the first report on the cytotoxic activity of hyperenone A (**4**) and 1,5-dihydroxy-2-methoxyxanthone (**6**).

4. Conclusion

Following a bioassay guided fractionation of the leave extract of *H. riparium*, twenty-three compounds were isolated and three of them were new. The extract and fractions showed antibacterial and cytotoxic activities against *S. aureus* and the human gastric cell line (BGC-823). Compounds **1**–**4**, **7** and **8** displayed moderate antibacterial activities. Additionally, compounds **4** and **6** showed inhibitory effects on the proliferation of the human gastric cell line (BGC-823). The overall results may support the use of this plant in traditional medicine for the treatment of gastric disorders and microbial infections.

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