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Two polymethoxylated flavonoids with antioxidant activities and a rearranged clerodane diterpenoid from the leaf exudates of *Microglossa pyrifolia*

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ABSTRACT

Three novel compounds; two polymethoxylated flavonoids, 5,7,4′-trihydroxy-3,8,3′,5′-tetramethoxyflavone (**1**), 5,7,3′-trihydroxy-3,8,4′,5′-trimethoxyflavone (**2**), and a clerodane diterpenoid; 8-acetoxyisochiliolide lactone (**3**) were characterized from the leaf exudates of *Microglossa pyrifolia*. In addition, three known polymethoxylated flavonoids including; 5,7,4′-trihydroxy-3,8,3′-trimethoxyflavone (**4**), 5,3′4′-trihydroxy-3,7,8-trimethoxyflavone (**5**), 5,3′4′-trihydroxy-7-methoxyflavanone (**6**) and a clerodane diterpenoid; 7,8-epoxyisocholiolide lactone (**7**) were identified. Their structures were determined on the basis of spectroscopic evidence. All the compounds did not exhibit antiplasmodial and antimicrobial activities at 47.6 μg/mL and were not cytotoxic at 5 μg/mL. Compound **6** exhibited modest antileishmanial activity with IC_{50} value of 13.13 μg/mL with **5** and **7** showing activities with IC_{50} values of 31.13 and 38.00 μg/mL, respectively, therefore inactive. The flavonoids (quercetin derivatives, **4** and **5**) showed similar antioxidant activities, using 2,2-diphenylpicrylhydrazyl (DPPH) assay, with IC_{50} values of 6.2 ± 0.3 μg/mL for **4** (17.3 μM) and **5** (17.8 μM) respectively. These activities were comparable to that of the standard quercetin (IC_{50} value of 6.0 ± 0.2 μg/mL (19.9 μM)), irrespective of methylation of the characteristic hydroxyl groups expected to be responsible for activity and additional substitution at C-8 in ring A of the flavonoid ring. These studies revealed that the presence of an hydroxyl group at C-4′ positions and oxygenation at C-3 in flavone skeleton, appears to be necessary for good antioxidant activities as encountered in compounds **1**, **4** and **5**. Substantial reduction in antioxidant activity was shown by methoxylation of the 4′-OH as observed in compound **2** with an IC_{50} value of 8.79 ± 0.3 μg/mL (24.4 μM).

1. Introduction

Microglossa pyrifolia (Lam.) Kuntze (Asteraceae, tribe Astereae) is a climbling shrub, softly hairy, growing to about 2 metres tall. It is indigenous to southern and tropical Africa and Asia (Neuwinger, 1994). In Kenya and Ghana it is used in traditional medicine to manage malaria and to reduce malaria related fevers (Abbiw, 1990; Köhler et al., 2002). It is also used as an abortifacient, analgesic, antipyretic and to treat microbial infections (Neuwinger, 1994; Köhler et al., 2002). Previous phytochemical investigations of different parts of this plant have yielded terpenoids, aurone glucosides, dihydrobenzofurans, polyacetylenes (Zdero et al., 1990a; Ruecker et al., 1992; Köhler et al., 2002; Schmidt et al., 2003). The extracts of the aerial parts and the root are reported to show good antiplasmodial activities (Köhler et al., 2002). Due to the tomentose nature of the aerial parts and the ashen look of the leaves of the plant, there was speculation of the existence of surface compounds in the glandular trichomes (Midiwo et al., 2007) and hence the motivation to study the leaf exudates of this plant. This paper reports the phytochemistry and antiplasmodial, antimicrobial, antileishmanial, antioxidant and cytotoxic activities of two new polymethoxylated flavonoids namely; 5,7,4′-trihydroxy-3,8,3′,5′-tetramethoxyflavone (**1**), 5,7,3′-trihydroxy-3,8,4′,5′-trimethoxyflavone (**2**) and one new clerodane diterpenoid 8-acetoxyisochiliolide lactone (**3**) along with four known ones.

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2. Results and discussion

2.1. Structure elucidation of new compounds

Chromatographic purification of the surface exudates of the leaves of *M. pyrifolia* led to the isolation of two new polymethoxylated flavonoids namely; 5,7,4′-trihydroxy-3,8,3′,5′-tetramethoxyflavone (**1**), 5,7,3′-trihydroxy-3,8,4′,5′-trimethoxyflavone (**2**) and one new clerodane diterpenoid namely; 8-acetoxyisochiliolide lactone (**3**) along with three known polymethoxylated flavonoids including; 5,7,4′-trihydroxy-3,8,3′-trimethoxyflavone (**4**) (Labbe et al., 1991; Horie et al., 1998), 5,3′4′-trihydroxy-3,7,8-trimethoxyflavone (**5**) (Henrick and Jefferies, 1965), 5,3′4′-trihydroxy-7-methoxyflavanone (**6**) (Ibrahim et al., 2003) and a clerodane diterpenoid; 7,8-epoxyisocholiolide (**7**) (Zdero et al., 1990a). Characterization of the new compounds (**1**, **2** and **3**) using a combination of spectroscopic techniques will be discussed below.

Compound **1** was isolated as yellow armophous solids. The ^{1}H NMR (δ_H 12.39, for chelated OH), ^{13}C NMR (δ_C 155.3 for C-2, 138.4 for C-3, 178.4 for C-4) NMR and mass (M^+ *m*/*z* 390, $C_{19}H_{18}O_9$) spectral data of compound **1** were consistent with a 5-hydroxyflavonol derivative (Mabry et al., 1970; Agrawal, 1989). Its molecular formula ($C_{19}H_{18}O_9$) was confirmed from its HR-EIMS (*m*/*z* 389.1028, calculated 390.3408). The ^{1}H NMR spectrum (Table 1) of this compound displayed two singlets at δ_H 6.27 (1H, *s*) and δ_H 7.42 (2H, *s*). The singlet proton at δ_H 6.27 showed HMQC correlation with carbon 6 resonating at δ_C 99.3 and HMBC cross peaks with C-5 (δ_C 156.3), C-7 (157.5), C-8 (127.9) and C-10 (104.4) suggesting the assignment of this proton to H-6. The placement of this proton at C-6 and not C-8 in ring A was ruled out because of the HMBC correlation of the chelated proton at 12.39 with the C-6 carbon. The singlet appearing at δ_H 7.42 as a result of two equivalent protons was assigned to H-2′ and 6′. The placement of the two protons (δ_H 7.42) at this position was confirmed from the HMBC cross peaks with C-6′ (δ_C 106.1), C-1′ (δ_C 120.1), C-3′/5′(148.2), C-4′ (δ_C 139.4) and C-2 (δ_C 155.3).

Furthermore, the ^{1}H NMR also displayed two sharp singlets integrating for 3 protons each at δ 3.83 3.84 and one at 3.85 (for two methoxyl groups) which showed HMQC correlations with the corresponding carbons at δ 60.1, 61.3 and 56.4 (for two equivalent carbons), respectively. Two of these methoxyl groups were placed at C-3 and C-8 due to the fact that they resonated at δ 61.3 and δ 60.1, respectively, which is typical for di-*ortho* substituted methoxyl groups expected to appear above δ_C 59 due to steric crowding (Panichpol and Waterman, 1978). The other two identical methoxyls were placed at C-3′and at C-5′. The placement of the methoxyl groups at δ 3.83, 3.84 and 3.85 to C-3, 8, 3′ and 5′ respectively, was confirmed from the HMBC correlation between the methoxyl protons and C-3 (δ_C 138.4), C-8 (δ_C 127.9), C-3′ (δ_C 148.2) and C-5′(δ_C 148.2), respectively. The spectral data of this compound are in close agreement with that reported in literature (Roitman and James, 1985) for 5,7-dihydroxy-3,4′,8-trimethoxyflavone (**8**), especially the ^{13}C NMR data for C-6, C-8 and C-9. From these spectral data and comparison with literature values, this compound was identified as 5,7,4′-trihydroxy-3,8,3′,5′-tetramethoxyflavone (**1**). This is the first report of this compound in nature.

Compound **2**, obtained as yellow armophous solids, was assigned the molecular formula $C_{19}H_{18}O_9$ from its HR-EIMS (*m*/*z* 389.1187, calculated 390.3408). The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1** except for the position of the methoxy and hydroxyl groups at C-3′and C-4′ in ring B, which are interchanged. The ^{1}H NMR spectrum showed a singlet at δ 6.13 (1H) which was assigned to H-6 on the *tri*-substituted ring A. The spectrum further indicated the presence of two *meta* coupled aromatic protons at δ_H 7.35 and 7.21 (*d*, *J* = 2.0 Hz) assigned to H-2′ and H-6′ of ring B, respectively. The assignment of these protons in the respective positions in the ring was confirmed from the HMBC correlations of the proton at δ_H 7.35 with C-2, C-3′, C-4′, C-6′ and that at δ_H 7.21 with C-2, C-1′, C-2′, C-4′ and C-5′. The ^{1}H NMR spectrum further displayed signals for four methoxyls at δ_H 3.84, 3.80 and 3.77 (for two methoxyls), three of which were di-*ortho* substituted, at δ 60.7, 60.3 and 60.2 from their HMQC correlation. The isolated carbon appeared at δ_C 56.1. The placement of the methoxyl groups at C-3, C-8, C-4′ and C-5′ was confirmed from the HMBC correlations of these protons with the carbons at δ_C 138.5/139.1, 129.1, 138.5/139.1 and 153.4/153.8. Based on the above spectroscopic evidence and reference to literature data the compound was identified to be 5,7,3′-trihydroxy-3,8,4′,5′-trimethoxyflavone (**2**). This is the first report of this compound in nature.

Compound **3**, obtained as white crystals, was assigned the molecular formula $C_{22}H_{26}O_7$ from its HR-EIMS (*m*/*z* 402.1700, calculated for 402.4376). The ^{13}C NMR spectrum corroborated the

Table 1
^{1}H (400 MHz) and ^{13}C NMR (100 MHz) NMR data along with important HMBC (^{2}J and ^{3}J) correlations of **1** and **2**.

Position	**1** ($CDCl_3$)			**2** ($CDCl_3$)		
	δ_H (*m*, Hz)	δ_C	HMBC	δ_H (*m*, Hz)	δ_C	HMBC
2		155.3			153.4/153.8	
3		138.4			138.5/139.1	
4		178.4			177.5	
5		156.3			156.7	
6	6.27 (*s*)	99.3	C-5, 8, 10	6.13 (*s*)	101.2	C-5, 8, 10
7		157.5			165.0	
8		127.9			129.1	
9		149.0			149.0	
10		104.4			101.7	
1′		120.1			126.0	
2′	7.42 (*s*)	106.1	C-2, 3′, 4′, 6′	7.35 (*d*, *J*=2)	110.2	C-2, 3′, 4′, 6′
3′		148.2			151.5	
4′		139.4			138.5/139.1	
5′		148.2			153.4/153.8	
6′	7.42 (*s*)	106.1	C-2, 1′, 2′, 4′, 5′	7.21 (*d*, *J*=2)	103.5	C-2, 2′, 4′, 5′
5 (OH)	12.39 (*s*)		C-5, 6, 10			
3-OMe	3.83 (*s*)	60.1	C-3	3.77 (*s*)	60.2/60.3/60.7	C-3
8-OMe	3.84 (*s*)	61.3	C-8	3.80 (*s*)	60.2/60.3/60.7	C-8
3′-OMe	3.85 (*s*)	56.4	C-3′			
4′-OMe				3.77 (*s*)	60.2/60.3/60.7	C-4′
5′-OMe	3.85 (*s*)	56.4	C-5′	3.84 (*s*)	56.1	C-5′

presence of twenty two carbon atoms suggesting a modified diterpenoid skeleton. The ^{1}H NMR spectrum of **3** showed the presence of three methyl groups resonances at δ_H 1.08 (3H, *s*), 1.13 (3H, *s*), 1.93 (3H, *s*) and an acetoxy methyl at 1.79 (3H, *s*). Furthermore, this spectrum displayed peaks for a β-substituted furan moiety as indicated by the characteristic doublets resonating at δ_H 6.45 (*d*, 1.5 Hz) and 7.47 (*d*, 1.5 Hz) and a singlet proton at 7.48 (*s*) assigned to the olefinic protons of the furan ring at H-14, H-15 and H-16, respectively. The ^{1}H NMR spectrum also exhibited a double doublet at 5.37 (1H, *dd*, 8.8, 8.2 Hz) assigned to the downfield shifted oxymethine proton at C-12 and a set of double doublets at δ_H 2.69 (H-11α) and 2.61 (H-11β) suggestive of a 20,12-olide. The ^{1}H and ^{13}C NMR spectral data of compound **3** supported the presence of two lactone carbonyls (δ_C 177.5 (C-3), 172.5 (C-20)) and an acetoxy carbonyl at δ_C 169.6 (C-21) as well as a furanyl ring (δ_C 144.5, 140.0, 126.0 and 108.5). The structure of **3** was confirmed from the HMBC experiment in which the olefinic protons at δ_H 7.48 (C-16) showed correlations with C-13 (δ 126.0) C-14 (δ 108.5), C-12 (δ 70.8). Similarly, the proton at δ_H 5.37 (H-12) showed cross peaks with C-9 (δ 54.4), C-14 (δ 108.5) and C-16 (δ 140.0). The methyl resonating at δ_H 1.79 exhibited HMBC correlations with the acetoxy carbonyl at δ_C 169.6 hence confirming its placement to C-21 in the clerodane skeleton. The presence of four olefinic carbons for a furanyl ring, two lactone carbonyls and three methyls suggests a clerodane diterpenoid parent skeleton (Zdero et al., 1990a). Furthermore, the ^{1}H and ^{13}C NMR spectral data matched those reported for the pentacyclic diterpenoid; 8-hydroxyisochiliolide lactone reported previously from *M. pyrrhopappa* (Zdero et al., 1990b). Based on the above spectroscopic evidence the compound was determined to be a novel compound which was named as; 8-acetoxyisochiliolide lactone (**3**).

2.2. Bioactivity

Compounds **1**–**7** were screened for their antiplasmodial activities against two strains of *Plasmodium falciparum*, antibacterial activities against *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa* and *Mycobacterium intracellulare bacteria* and antifungal activities against *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Cryptococcus neoformans*, *Aspergillus fumigatus* fungal strains, antileishmanial activities against *Leishmania donovanii*, cytotoxic activities against Vero cells and antioxidant potential using DPPH reagent.

All the compounds did not exhibit antiplasmodial and antimicrobial activities at the initial concentration of 47.6 μg/mL and were not cytotoxic up to the maximum concentration tested (5 μg/mL). However, compound **6** exhibited modest antileishmanial activity with IC_{50} value of 13.13 μg/mL while **5** and **7** showed activities with IC_{50} values of 31.13 and 38.00 μg/mL respectively, therefore inactive.

Flavonoids (quercetin derivatives, **4** and **5**) showed similar antioxidant activities, using 2,2-diphenylpicrylhydrazyl (DPPH) assay, which were comparable to that of 3,5,7,3′,4′-pentahydroxyflavanol (quercetin) irrespective of methylation of the characteristic hydroxyl groups (3-OH and 3′-OH) and additional substitution at C-8 (8-OCH_3) in ring A of the flavonoid ring. Methylation of the hydroxyl groups at 3, 3′ and 7 has minimal influence on activity as shown in compounds **1**, **4** and **5** with IC_{50} values of 6.45 ± 0.3 (16.5 μM) and 6.2 ± 0.3 μg/mL (for **4** (17.3 μM) and **5** (17.8 μM) respectively. However, an additional methoxyl group at 5′ position in ring B of compound **4** resulted in minimal reduction in activity as shown in compound **1** with an IC_{50} value of 6.45 ± 0.3 μg/mL. Substantial reduction in antioxidant activity was shown by methoxylation of the 4′-OH as observed in compound **2** (IC_{50} value of 8.79 ± 0.3 μg/mL (24.4 μM)), with an oxygenation pattern similar to **1** except for the position of the methoxy and hydroxyl groups at C-3′and C-4′ in ring B, which are interchanged. From these observations it is not clear whether the reduction in activity of compounds **4** and **5** with reference to quercetin which exhibited an activity of 6.0 ± 0.2 μg/mL (19.9 μM) was due to the additional oxygenation at C-8 in ring A or methoxylation of the C-3, C-5′and C-7 hydroxyl groups. However, previous studies have shown that methylation of the 3-OH in flavanols substantially reduced antioxidant activities compared to methylation of the hydroxyl groups at the 5-OH, 7-OH, 4′-OH and 3′-OH of quercetin (Op de Beck et al., 2003; Teffo et al., 2010). The flavones **2**, lacking a free hydroxyl group at C-4′ and the flavanone (**6**) lacking oxygenation at 3′ position exhibited substantially lower antioxidant activity with IC_{50} values of 8.79 ± 0.3 (24.4 μM and 8.54 ± 0.3 μg/mL (28.3 μM), respectively. These studies revealed that the presence of an hydroxyl group at C-4′ positions and oxygenation at C-3 in flavone skeleton, appears to be necessary for good antioxidant activities as encountered in compounds **1**, **4** and **5**. Further structure activity relationships studies are required to comprehensively establish the relationship between the oxygenation pattern, substitution in the flavonoid ring and the antioxidant activities.

3. Experimental

3.1. General experimental procedures

Column chromatography was carried out using Merck silica gel 40 (70–230 mesh) and Fluka Sephadex LH-20 as stationary phases. Analytical TLC and Preparative TLC were done using Merck precoated 60 F_{254} and Merck 60 PF_{254} respectively. Compounds were visualized by observing under UV light at 254 or 365 nm, followed by spraying with 1% vanillin-H_2SO_4 spray reagent and placing the plates in iodine tanks to view the spots that were not UV active. 1D and 2D NMR spectra were recorded in $CDCl_3$ on a 400 MHz Bruker AVANCE NMR instrument at room temperature. Chemical shifts, δ, were expressed in ppm and referenced against the solvent resonances at 7.26 and 77.23 ppm for ^{1}H and ^{13}C NMR respectively. Electron ionization mass spectroscopy (EIMS) spectra were recorded on 70 eV, on SSQ 710 MAT mass spectrometer. UV values were obtained using SP8 150 ultra violet visible (UV/VIS) spectrophotometer.

3.2. Plant material

The fresh leaves of *M. pyrifolia* were collected from Ngong forest (about 6 km from Nairobi city centre, Kenya), on 17th August, 2012, and identified by Mr. Patrick Mutiso of the University of Nairobi Herbarium, School of Biological Sciences (SBS), where voucher specimen (Akimanya-07/August, 2012) is deposited.

3.3. Extraction and isolation of compounds from the leaves of M. pyrifolia

The fresh leaves (7.5 kg) of *M. pyrifolia* were extracted by successive dipping into fresh portions of ethyl acetate for short periods (ca. 15 s) to avoid extraction of internal tissue compounds such as chlorophyll. The extracts obtained were filtered under pressure and solvent removed *in vacuo* using a rotary evaporator resulting to 180 g (2.4% yield) of light green yellow crude extract. A portion of the crude extract (150 g) was dissolved in 2% dichloromethane (CH_2Cl_2) in methanol (MeOH) and adsorbed on silica gel (150 g, Merck Grade 9385, pore size 60 Å, 230–400 mesh particle size). The adsorbed silica gel was loaded onto a column (15 cm × 100 cm) packed with silica gel (150 × 10 g) under 50% CH_2Cl_2 in normal hexane (n-C_6H_{12}). Separation was carried out by stepwise gradient elution with mixtures of CH_2Cl_2 in n-C_6H_{12} up to

100% and then with MeOH in CH_2Cl_2 up to 10% in increasing order of polarities; leading to 98 fractions of 200 mL each. The fractions were concentrated and spotted on an analytical TLC plate, where fractions with similar profiles were combined leading to 20 fractions only (A–L). White crystals of epoxyisochiliolide lactone (**7**, 86 mg) precipitated out of the fraction of the major column eluted with 30% CH_2Cl_2 in *n*-C_6H_{12}. The solids were filtered, dried and purified further through recrystallization in the same solvent system. The combined fraction eluted with (40–50% CH_2Cl_2 in *n*-C_6H_{12}, 1.9 g) was subjected to column chromatography (20 g, 2.0 cm × 60 cm column diameter and height) on silica gel and with stepwise gradient elution with *n*-C_6H_{12} with increasing amounts of CH_2Cl_2 up to 100%, resulting to 10 fractions of 100 mL each. The fraction of the minor column eluted with 50% CH_2Cl_2 in *n*-C_6H_{12}, yielded 8-acetoxyisochiliolide lactone (**3**, 210 mg) after passing it through Sephadex-LH 20. The fraction from the major column eluted with 60% CH_2Cl_2 in *n*-C_6H_{12} (120 mg) was concentrated *in vacuo*, dissolved in minimum quantities of MeOH:CH_2Cl_2; 1:1 v/v) and loaded onto a Sephadex-LH 20 and eluted with the same solvent to yield two major fraction of 400 mL each. The second fraction of this column yielded yellow crystals which were recrystallized from *n*-C_6H_{12}/CH_2Cl_2 (1:1) to give 5,3′,4′-trihydroxy-3,7,8-trimethoxyflavone (**5**, 47 mg). The fraction from the major column eluted with 80% CH_2Cl_2 in *n*-C_6H_{12}, (K, 11.04 g) was purified further by column chromatography using silica gel (102 g, 4 × 80 cm) eluting with increasing gradient of CH_2Cl_2 in *n*-C_6H_{12} up to 100% and then MeOH in CH_2Cl_2 up to 5%. The eluants were collected in 100 mL Erlenmeyer flasks leading to 30 fractions. TLC (2% MeOH in CH_2Cl_2) analysis of fraction 8–30 of this minor column showed similar profiles and therefore were combined. Removal of the solvent of the combined fractions and recrystallization (80% CH_2Cl_2 in *n*-C_6H_{12}) afforded yellow needles of 5,7,4′-trihydroxy-3,8,3′-trimethoxyflavone (**4**, 80 mg). The mother liquor was concentrated and spotted onto a TLC plate resulting only two major spots. Purification of the mother liquor of the above fractions using PTLC (silica gel, 100% CH_2Cl_2 multiple development) afforded 5,7,4′-trihydroxy-3,5,8,3′-tetramethoxyflavone (**1**, 46 mg) and 5,3′,4′-trihydroxy-7-methoxyflavanone (**6**, 35 mg). The fraction of the major column eluted with 2% MeOH in CH_2Cl_2 (16.76 g) was chromatographed on a silica gel column (200 g, 4 × 80 cm) eluting with increasing gradient of EtOAc in *n*-C_6H_{12} up to 100% EtOAc to afford 20 fractions of 200 mL. The fraction eluted with 50% EtOAc in *n*-C_6H_{12} after purification with Sephadex-LH 20 (MeOH in CH_2Cl_2; 1:1 v/v) yielded yellow crystals which were purified by recrystallization (2% MeOH in CH_2Cl_2) to yield 5,7,3′-trihydroxy-3,8,4′,5′-trimethoxyflavone (**2**, 52 mg) in the second to fifth fraction of 200 mL each.

3.4. 5,7,4′-Trihydroxy-3,8,3′,5′-tetramethoxyflavone (*1*)

Yellow armophous solids. Melting point 172–172.5 °C. ^{1}H and ^{13}C NMR (Table 1); HRMS (*m*/*z*, rel. int.): HRMS found *m*/*z* 389.1028 (*calcd.* for $C_{19}H_{18}O_9$, 390.3408).

3.5. 5,7,3′-Trihydroxy-3,8,4′,5′-trimethoxyflavone (*2*)

Yellow armophous solids. Melting point 172.5–173.2 °C. ^{1}H and ^{13}C NMR (Table 1); HRMS (*m*/*z*, rel. int.): HRMS found *m*/*z* 389.1189 (*calcd.* for $C_{19}H_{18}O_9$, 390.3408).

3.6. 8-Acetoxyisochiliolide lactone (*3*)

White crystals. Melting point 177–178 °C ^{1}H and ^{13}C NMR (Table 2); HRMS (*m*/*z*, rel. int.): HRMS found *m*/*z* 402.1700 (*calcd.* for $C_{22}H_{26}O_7$, 402.4376).

Table 2
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) NMR data along with important HMBC (^2J and ^3J) correlations of **3**.

Position	**3** ($CDCl_3$)		
	δ_C (*m*, Hz)	δ_H	HMBC
1	28.8	2.23 (*ddd*, 13.6, 10.5, 3.8); 1.84 (*ddd*, 13.6, 9.2, 4.2)	C-4, 5, 9, 10
2	29.4	1.94 (*m*); 1.71 (*ddd*, 13.0, 9.2, 3.8)	C-3, 5, 10
3	177.5		
4	54.4		
5	51.2		
6	26.1	1.44 (*td*, 14.0, 3.2); 1.39 (*dt*, 14.0, 3.8)	C-4, 7, 8, 10
7	31.5	2.54 (*dt*, 14.0, 3.6); 1.75 (*m*)	C-5, 8, 9, 17
8	86.0		
9	54.4		
10	93.3		
11	34.4	2.69 (*dd*, 13.8, 8.2); 2.61 (*dd*, 13.8, 8.8)	C-8, 10, 13, 20
12	70.8	5.37 (*dd*, 8.8, 8.2)	C-9, 13, 14, 16
13	126.0		
14	108.5	6.45 (*d*, 1.5)	C-12, 13, 15, 16
15	144.5	7.47 (*d*, 1.5)	C-13, 14
16	140.0	7.48 (*s*)	C-12, 13, 14
17	21.2	1.93 (*s*)	C-7, 9
18	9.4	1.08 (*s*)	C-3, 5
19	16.9	1.13 (*s*)	C-4, 6
20	172.5		
$CH_3\underline{C}OO$–	169.6		
$\underline{CH_3}COO$–	22.2	1.79 (*s*)	C-21

3.7. In vitro *antiplasmodial activity assay*

Antiplasmodial activities of the crude extracts and pure compounds were determined on two strains *of P. falciparum*; Sierra Leone D6 (chloroquine-sensitive) and Indochina W2 (chloroquine-resistant) using the modified assay described by Trager and Jensen (1976), also used by Makler and Hinrich (1993) and Samoylenko et al. (2009). DMSO, artemisinin (98% purity assessed by HPLC) and chloroquine (98% purity assessed by HPLC) drugs were used as controls. Initially, a concentration of 47.6 μg/mL was used for the crude extracts and those extracts showing ≥50% inhibition were selected for further testing. The selected extracts and enriched fractions were dissolved to 20 mg/mL and tested at 47.6, 15.9, and 5.3 μg/mL. The pure compounds (≈95% purity assessed by NMR and MS) and some enriched fractions were dissolved to 2 μg/mL and tested at 4.76, 1.59, and 0.53 μg/mL.

3.8. In vitro antileishmania activity assay

In vitro antileishmanial activities of the compounds were done on a culture of *L. donovanii* promastigotes (2 × 106 cells/mL) in a 96-well-microtiter-plates with an initial concentration of 80 μg/mL and subsequent 4 fold serial dilution. The plates were then incubated at 26 °C for 72 h and their growth monitored by Alamar blue assay (Mikus and Steverding, 2000). Pentamidine (≥95% purity assessed by HPLC) and amphotericin B (≈80% purity assessed by HPLC) were used as positive control and the IC_{50} values computed from the growth inhibition curve.

3.9. In vitro *antimicrobial activity assay*

The antimicrobial susceptibility assays were performed using a modified CLSI method as described by Samoylenko et al. (2009). Ciprofloxacin (≥98% purity assessed by HPLC, ICN Biomedicals, OH) for bacteria and amphotericin B (≈80% purity assessed by HPLC, ICN Biomedicals, Ohio) for fungi were used as positive controls. The test organisms were obtained from the American Type Culture Collection, ATCC (Manassas, VA) and included *C. albicans* (ATCC 90028), *C. glabrata*

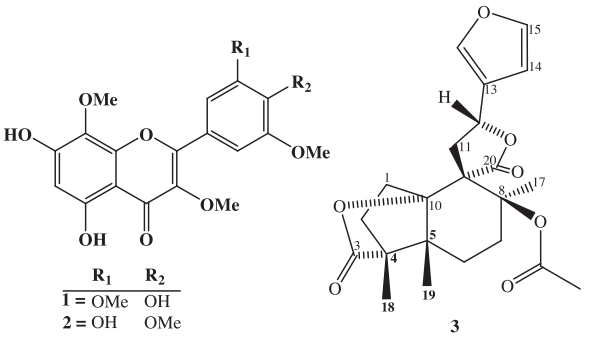

Fig. 1. Chemical structures of compounds **1**–**3**.

(ATCC 90030), *C. krusei* (ATCC 6258), *A. fumigatus* (ATCC 90906), *C. neoformans* (ATCC 90113) fungi and *S. aureus* (ATCC 29213), methicillin-resistant *S. aureus* (ATCC 33591), *E. coli* (ATCC 35218), *P. aeruginosa* (ATCC 27853) and *M. intracellulare* (ATCC 23068) bacteria.

4. *In vitro* cytotoxicity analysis

The cell viability studies were done against monkey kidney fibroblasts (VERO) obtained from the American Type Culture Collection (ATCC, Rockville, MD). The assays were performed in 96-well microplates with the cells seeded at a density of 25,000 cells/well and incubated for 24 h. Samples at different concentrations were added and plates were further incubated for 48 h. The number of viable cells was determined using Neutral Red according to a modified procedure of Borenfreund et al. (1990). Doxorubicin (98–102% purity assessed by HPLC) was used as a positive control and DMSO as the negative control.

4.1. Antioxidant activity tests

Preliminary anti-oxidant test was done by spotting the compounds on a TLC plate followed by spraying it with 0.2 mg/mL DPPH solution to observe the active compounds which discharged the purple colour of DPPH to white or yellow. The active compounds were quantitatively analyzed by UV–vis spectrometry method adopted by Hou et al. (2002) with modifications on the concentrations of samples. For each active compound, the concentration of the sample was varied by serial dilutions to give concentrations of 160, 80, 40, 20, 10, 5.0, 2.5, 1.25 μg/mL while the concentration of DPPH was kept constant at 100 μg/mL (Fig. 1). The tests were done in triplicate. These solutions were then measured for UV–vis absorbance at DPPH absorbing wavelength (517 nm) half an hour after adding the DPPH solution. The absorbance measured at each of these concentrations were converted into percentage radical scavenging activity (RSA) calculated as follows;

$$\mathrm{RSA}\,(\%) = \frac{A_{\mathrm{blank}} - A_{\mathrm{sample}}}{A_{\mathrm{blank}}} \times 100$$

where A_{blank} refers to the optical density (OD) of the control (DPPH solution) while A_{sample} refers to the OD of the test sample (Erasto et al., 2011). The percentages of scavenged DPPH were then plotted against concentration of the compound to give graphs from which concentrations at half inhibition (IC_{50}) were determined.

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