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Flavonols and an oxychromonol from *Piliostigma reticulatum*

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ABSTRACT

The leaf extract from the plant *Piliostigma reticulatum* was found to exhibit antimicrobial activity against some bacteria and fungi such as *Staphylococcus aureus* (NCTC 6571), *Escherichia coli* (NCTC 10418), *Bacillus subtilis* (NCTC 8236), *Proteus vulgaris* (NCTC 4175), *Aspergillus niger* (ATCC 10578) and *Candida albicans* (ATCC 10231). Upon investigation of the chemical constituents present in the leaf extract, a total of seven compounds were isolated and their structures were unambiguously established by spectroscopic methods including HR-MS and NMR spectrometry. Four of the isolated compounds were novel, namely **6**-*C*-methyl-2-*p*-hydroxyphenyloxychromonol (piliostigmol), **1**, 6,8-di-*C*-methylquercetin-3,3′,7-trimethyl ether, **2**, 6,8-di-*C*-methylquercetin-3,3′-dimethyl ether, **3** and 3′,6,8,-tri-*C*-methylquercetin-3,7-dimethyl ether, **4**. The other three were known C-methylated flavonols and they were isolated from *P. reticulatum* for the first time. These were 6-*C*-methylquercetin-3-methyl ether, **5**, 6,8-di-*C*-methylkaempferol-3-methyl ether, **6** and 6-*C*-methylquercetin-3,3′,7-trimethyl ether **7**. All the isolated compounds were tested for cytotoxicity using the brine shrimp toxicity assay and all of them were active albeit at different levels. With respect to antibacterial activity piliostigmol, **1** showed the highest activity against E. coli (MIC = 2.57 μg/ml, 0.006 μmol), which is three times more that of Amoxicillin, where as **4** and **7** showed the least activity.

1. Introduction

Piliostigma reticulatum is a leguminous plant belonging to the family Caesalpiniaceae, a family that comprises of trees, shrubs or very rarely climbers. The tree is perennial in nature and its vernacular names include Abefe, Monkey bread, Camel's foot, Kalgo and Okpoatu and these vary according to locality. *P. reticulatum* has a close resemblance to P. *thonningii* to such an extent that they may easily be confused with each other, although when examined very closely, distinguishing morphological features become readily observable. *P. reticulatum* has smaller leaves and smoothness on the lower side whereas *P. thonningii* has larger leaves and hairiness on the lower side. *P. reticulatum* is widely distributed in Africa and Asia (Djuma, 2003; Schultes and Hofmann, 1973). Ethnomedically, the bark, root, pod, young stem or leaves have been used for treating leprosy, smallpox, coughs, ulcer, heart pain, gingivitis, snake bite, dysentery, fever, wounds and a variety of closely related disease conditions (Irvine, 1961; Asuzu and Onu, 1994; Bombardelli et al., 1973, 1992, 1994; Dalziel, 1937; Watt and Breyer-Brandwijk, 1962; McGaw et al., 1997; Okwute et al., 1986). Previously isolated constituents include flavonoids and proanthocyanidins (Snatzke and Wolff, 1989; Lamikanra et al., 1990; Komiya et al., 1975), labdane derivatives (Hashidoko et al., 1991; Huang et al., 1993), a kaurane diterpene (Roitman and James, 1985; Martin et al., 1997), *C*-methylflavonols (Breitmeier and Voelter, 1990; Harborne et al., 1994; Rabesa and Voirin, 1985; Voirin, 1983; Aderogba et al., 2003; Rasamoelisendra et al., 1989) and phenoxychromones (Ibewuike et al., 1996, 1997), while broad phytochemical analysis showed the presence of other classes of constituents such as saponins, tannins and polyphenols (Irvine, 1961; Bombardelli et al., 1994).

2. Results and discussion

Dried powdered leaves were subjected to sequential extraction with *n*-hexane, ethyl acetate and methanol. Fractionation of the resulting extracts on silica gel as well as Sephadex LH 20 chromatography led to the isolation of compounds **1–7**.

The mass spectrum of **1** showed a molecular ion base peak $(M)^{+\cdot}$ at *m/z* 330.0681 and this together with the mass spectral fragmentation suggested a molecular formula of $C_{17}H_{14}0_7$. The UV spectrum exhibited two peak maxima at 232 and 287 nm, which were in close agreement with those obtained for capillarisin, a closely related 2-*O*-(*p*-hydroxyphenyl)chromone, which was isolated

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from *Artemisia capillaris* (Komiya et al., 1975), thus indicating the possible presence of a similar chromophore in **1**. The ^{1}H NMR spectrum showed a characteristic pattern for an A_2B_2 system, as would be expected from the *para*-substituted aromatic system of the phenolic substituent, with doublets at δ 6.85 and 7.19 (J = 8.8 Hz), as a well as a singlet at δ 6.72 which could be attributed to H-8 in the A ring (Komiya et al., 1975; Hashidoko et al., 1991; Huang et al., 1993). Other signals of note were a chelated 5-OH at δ 12.95, a singlet at 11.65 due to the 3-OH, a methyl singlet at δ 2.01 and a methoxyl singlet at δ 3.90. In the ^{13}C NMR spectrum, the signal due to C-3 appeared at δ 156.8 while that of C-8 was observed at δ 92.1 and this was corroborated by HMQC data. The observed HMBC correlations for **1** (Fig. 2) confirmed that the methine group was located at position 8 (cross peaks H-8/C-4, C-6, C-7, C-9, C-10), that the methyl group occurred as a substituent at C-6 (cross peaks OH-5/C-5, C-10 and Me-6/C-5, C-6, C-7) whereas the methoxyl group was linked to C-7 (cross peak OMe/C-7). No HMBC correlations were observed between rings B and C, thus confirming the presence of the oxygen bridge C1′–O–C2. Further evidence was also provided by the chemical shift of 166 ppm for C-2, which is about 11 ppm downfield to that of flavones, and which is in agreement with reported literature (Huang et al., 1993). All the above data is in agreement with the structure of **1** having been assigned as 2-*O*-(*p*-hydroxyphenyl)-3,5-dihydroxy-6-*C*-methyl-7-methoxychromone, and is hereby given the trivial name piliostigmol (Fig. 1).

Compounds **2**–**4** were similarly assigned based on their quercetin chromophore and respective spectral data.

Compound **2** showed a molecular ion peak $(M)^{+\cdot}$ at *m/z* 372.1103 leading to a molecular formula of $C_{20}H_{20}0_7$. The UV spectrum showed two peak maxima at 355 and 257 nm, respectively. The band at 270 nm showed a large bathochromic shift in the presence of sodium acetate, and an additional band appeared at 342 nm in the methanol/NaOH spectrum which suggested a flavonol with a 7-OMe group. The ^{1}H NMR spectrum showed two methyl singlets at δ 2.12 and 2.36 and three methoxyl peaks at δ 3.78 (3H, s, OMe-3), 3.97 (3H, s, OMe-3′) and 4.03 (3H, s, OMe-7). The spectrum further showed the presence of only three aromatic signals at δ 6.91 (1H, d), 7.60 (1H, dd) and 7.70 (1H, d) suggesting a 3′,4′-substituted aromatic ring C, and a fully substituted aromatic ring A. The characteristically chelated 5-OH group was observed at δ 12.92. Based on a comparison of the ^{13}C NMR data of **2** with existing literature (Roitman and James, 1985; Breitmeier and Voelter, 1990) it could be inferred that the 4′-substituent was a hydroxyl group while that at the 3′ position was a methoxyl group; this was further confirmed by the chemical shifts of the substituted carbons, C-3′ at δ 147.2 and C-4′ at 148.5. The other methoxyl groups were linked to C-3 (δ 137.5) and to C-7 (δ 162.4) which was in agreement with the HMBC correlations OMe-3/C-3 and OMe-7/C-7. The methyl groups were joined to C-6 and C-8, as implied by the HMBC cross peaks OH-5/C-5, C-6, C-10; Me-6/C-5, C-6, C-7 and that of Me-8/C-7, C-8, C-9. Based on the above information the structure of **2** was suggested to be 3,3′,7-trimethoxy-6,8-di-*C*-methyl-4′,5-dihydroxyflavone (6,8-di-*C*-methylquercetin 3,3′,7-trimethyl ether) (Fig. 3).

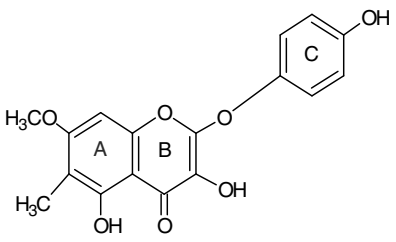


Fig. 1. (**1**) Piliostigmol.

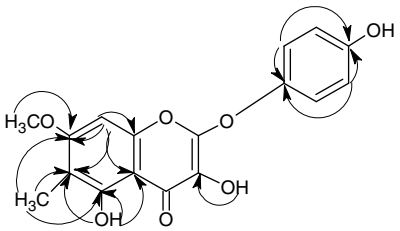
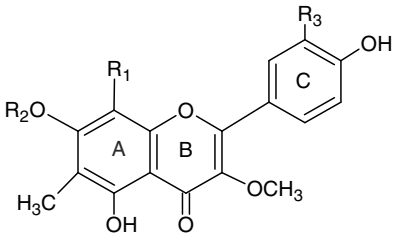


Fig. 2. (**1**) HMBC correlations for piliostigmol.



	R_1	R_2	R_3
2	CH_3	OCH_3	OCH_3
3	CH_3	OH	OCH_3
4	CH_3	OCH_3	CH_3
5	H	OH	OH
6	CH_3	OH	OH
7	H	OCH_3	OCH_3

Fig. 3. Compounds **2**–**7**.

The molecular formula of **3** was assigned as $C_{19}H_{18}0_7$ on the basis of the molecular ion peak at *m/z* 360.0954. The UV spectrum with maxima at 354 and 262 nm suggested a flavonol with a 7-OH group. The ^{1}H NMR spectrum exhibited signals attributable to two methyl groups at δ 2.11 (3H, s, Me-6) and 2.41 (3H, s, Me-8) and two methoxyl groups at 3.89 (3H, s, OMe-3) and 3.98 (3H, s, OMe-3′). The aromatic band area showed signals typical of a 3′,4′-substituted ring C similar to that observed for **2** at δ 7.03 (1H, d), 7.62 (1H, dd) and 7.75 (1H, d). The signals due to the three OH groups appeared at δ 10.23 (1H, s, 4′-OH), 12.95 (1H, s, 7-OH) and at 13.05 (1H, s, 5-OH). The ^{13}C spectrum (Table 1) showed a pattern similar to that of compound **2**, in which the C ring is substituted with one hydroxyl group at C-4′ and a methoxyl group at C-3′. The HMBC data for ring A was, as expected, somewhat different from that of **2**, with the C-7 position showed the presence of an OH group. The cross peaks observed for OH-5/C-5, C-6, C-10 and Me-6/C-5, C-6, C-7, Me-8/C-7, C-8, C-9 indicated that the methyl groups were located at C-6 and C-8 due to their respective coupling effects. The location of the two methoxyl groups at C-3 and C-3′ was further verified by their HMBC correlations with C-3 and C-3′, respectively. **3** was consequently assigned the structure 3,3′-dimethoxy-6,8-di-*C*-methyl-4′,5,7-trihydroxyflavone (6,8-di-*C*-methylquercetin 3,3′-dimethyl ether) (Fig. 3).

The EIMS of **4** showed the molecular ion peak $(M)^{+\cdot}$ at *m/z* 356.3821 corresponding to a molecular formula of $C_{20}H_{20}0_6$. The UV spectrum showed a pattern that was also similar to that of

Table 1
^{13}C NMR (100 MHz) data for compounds **1**–**4** in DMSO-d_6

	1	2	3	4
2	166.7	155.3	155.2	155.8
3	156.8	137.5	137.3	137.2
4	182.3	178.8	177.9	178.4
5	156.2	155.3	157.1	155.9
6	108.5	106.6	107.3	112.6
7	161.5	162.4	162.1	162.5
8	92.1	101.5	101.9	108.4
9	152.4	151.4	154.4	151.3
10	101.5	103.9	104.8	107.1
1′	143.1	121.6	120.4	120.5
2′	121.5	115.3	115.4	115.5
3′	116.2	147.2	147.6	135.6
4′	156.2	148.5	148.5	148.8
5′	116.6	115.8	115.4	115.6
6′	121.8	120.6	120.8	120.2
3-OMe		59.5	59.2	59.4
7-OMe	56.4	60.2		60.3
3′-OMe		56.5	55.9	
6-Me	7.2	8.1	7.2	8.0
8-Me		8.5	8.3	8.2
3′-Me				8.7

2 with maxima at 363 and 263 nm suggesting a flavonol with a 7-OMe group. The ^{1}H NMR spectrum exhibited signals of three methyl groups at δ 2.16 (3H, s, Me-6), 2.29 (3H, s, Me-3′) and 2.42 (3H, s, Me-8). It also showed two peaks due to methoxyl groups at 3.84 (3H, s, OMe-3) and 3.92 (3H, s, OMe-7). The aromatic band region showed signals typical of a 3′,4′-substituted ring C as was observed for **2**, at δ 7.02 (1H, d), 7.62 (1H, dd) and 7.74 (1H, d) as well as signals of two OH groups at δ 10.13 (1H, s, 4′-OH) and 13.02 (1H, s, 5-OH). The main difference between the NMR data of **4** and **2** was the occurrence in the former of the proton peak located at δ 2.29 due to Me-3′ and a carbon signal at 135.6 due to C-3′ (Table 1). It was also evident that ring A was fully substituted and carrying a hydroxyl group at C-5, singlet at δ 13.02 while the second hydroxyl group was located at C-4′, singlet at 10.13. There were three methyl groups with direct linkages to carbon atoms, the first (δ 2.16) located at C-6 and the second (δ 2.42) at C-8 and the third (δ 2.29) at C-3′. These assignments were further corroborated by HMBC correlations which resembled those of **2** and **3**. These results lead to the identification of **4** as 3 7-dimethoxy-3′,6,8-tri-*C*-methyl-4′,5-dihydroxyflavone (3′,6,8-tri-*C*-methylquercetin 3,7-dimethyl ether) (Fig. 3).

Examination of the spectroscopic data generated from **5**, **6** and **7** showed that they were essentially identical to those of compounds already reported in the literature (Harborne et al., 1994; Voirin, 1983; Rabesa and Voirin, 1985).

To our knowledge compounds **1–4** have not been reported previously whereas **5–7** have been isolated for the first time from *P. reticulatum*.

2.1. Brine shrimp toxicity assay

Bioactive compounds have on occasion been found to be toxic in high doses; hence their pharmacology may be preliminarily assessed on the basis of their toxicology results. Thus, the *in vivo* lethality test on a simple zoological organism, such as brine shrimp larvae (nauplii), has been used as a convenient tool for screening of bioactive natural products (Meyer et al., 1982). Using this system, natural product extracts, fractions or pure compounds may be tested at three concentration levels of 10,100 and 1000 ppm or μg/ml in vessels containing 10 nauplii, the tests being performed in triplicate for each concentration level. The number of survivors is determined after 24 h, and this is used to estimate the LC_{50} with 95% confidence using the Finney Probit analysis computer programme.

The toxicity bioassay was conducted on ten samples, namely, the three crude extracts (as derived from the solvents, hexane, EtOAc and methanol) and the seven isolates (**1–4** and **5–7**). The results (Table 2) showed that all the extracts and nearly all the isolates were active (AC) (LC_{50} < 1000 μg/ml), except **4** and **7**, which were inactive (IA). Compound **1** showed the highest activity (very

Table 2
Brine shrimp lethality test result

Test material	1000 μg/ml	100 μg/ml	10 μg/ml	LC_{50}	Remarks
The average number dead, counted after 24 h					
Hex	9	7	3	33.3913	AC
EtOAc	10	8	6	*	AC
MeOH	10	10	7	3.3774	VA
1	10	10	9	0.4336	VA
2	9	7	4	23.1515	AC
3	10	8	6	6.117	AC
4	5	3	1	989.29	IA
5	10	8	7	2.4050	VA
6	10	10	6	6.2525	AC
7	4	1	0	1756.39	IA

Test material (%)	Vial at 1000 μg/ml	% at 1000 μg/ml	Vial at 100 μg/ml	% at 100 μg/ml	Vial at 10 μg/ml	% at 10 μg/ml	Vial for control experiment
The average number of survivors counted after 24 h							
Hex	0.67	6.67	3.33	33.33	7.0	70.0	100
EtOAc	0.33	3.33	2.33	23.33	3.67	36.67	100
MeOH	0	0	0.33	3.33	2.67	26.67	100
1	0	0	0	0	1.33	13.33	100
2	0.67	6.67	3.33	33.33	6.0	60.0	100
3	0	0	1.67	16.67	4.33	43.33	100
4	5	50.0	7	70.0	8.67	86.67	100
5	0.33	3.33	1.67	16.67	3.33	33.33	100
6	0	0	0.33	3.33	4.0	40.0	100
7	6.0	60.0	8.67	86.67	10.0	100	100

The results were classified based on the regressional finnery probit analysis programme of the LC_{50} as very active (VA), active (AC) and inactive (IA) at the three test concentrations.
Hex, *n*-hexane extract; EtOAc, ethyl acetate extract; MeOH, methanol extract.
* Data did not converge and therefore could not be regressed by the finnery probit analysis programme.

Table 3
Minimum inhibitory concentrations (MIC) of the constituents of *Piliostigma reticulatum*

Compounds	*S. aureus*		*B. subtilis*		*E. coli*		*P. vulgaris*		*A. niger*		*C. albicans*	
	μg/ml	μmol	μg/ml	μmol	μg/ml	μmol	μg/ml	μmol	μg/ml	μmol	μg/ml	μmol
Hex	06.50	0.018	12.50	0.032	25.00	0.083	10.50	0.031	25.00	0.081	12.50	0.027
EtOAc	12.50	0.035	25.00	0.102	50.00	0.203	12.50	0.051	25.00	0.102	50.00	0.203
MeOH	12.50	0.025	20.00	0.067	25.00	0.074	12.50	0.035	25.00	0.072	10.50	0.038
1	03.25	0.008	NoT	NoT	02.57	0.006	06.50	0.018	20.00	0.063	10.50	0.033
2	10.50	0.037	20.00	0.058	12.50	0.033	20.00	0.076	12.50	0.028	25.00	0.102
3	06.50	0.015	10.50	0.034	25.00	0.075	12.50	0.038	25.00	0.075	20.00	0.058
4	50.00	0.156	25.00	0.075	200.0	0.443	100.0	0.321	NoT	NoT	200.0	0.413
5	10.50	0.034	12.50	0.037	25.00	0.076	12.50	0.033	25.00	0.088	50.00	0.161
6	12.50	0.036	NoT	NoT	12.50	0.035	25.00	0.072	20.00	0.061	12.50	0.037
7	300.0	0.479	–	–	–	–	–	–	–	–	300.0	0.483
Am	03.25	0.008	06.50	0.018	10.50	0.032	06.50	0.016	–	–	–	–
Flu	–	–	–	–	–	–	–	–	–	–	20.00	0.062

Hex, *n*-hexane extract; EtOAc, ethyl acetate extract; MeOH, methanol extract.
Am, amoxicillin standard; Flu, fluconazole; NoT, not tested.
Test organisms are: Gram-positive bacteria: *Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis* (*B. subtilis*).
Gram-negative bacteria: *Escherichia coli* (*E. coli*) and *Proteus vulgaris* (*P. vulgaris*).
Fungi: *Aspergillus niger* (*A. niger*) and *Candida albicans* (*C. albicans*).

active, VA) and it was active at all the concentration levels tested. Since the lethality of a test substance to brine shrimp nauplii has been linked to the possible ability of such substance to kill cancer cells (antitumor activity), as well as pesticidal and antibacterial activities (Meyer et al., 1982) it may be deduced that all the VA and AC samples should be good candidates for such applications. Some of the compounds merely slow down the activity of the nauplii and such compound types are usually suspected to have an adverse effect on the central nervous system (CNS) (Meyer et al., 1982; Aderogba et al., 2006).

2.2. Antimicrobial activity tests

The same samples used in the brine shrimp lethality assay above were subjected to antimicrobial tests. The results, which were generated based on the microdilution technique (Drummond and Waigh, 2000) are presented as minimum inhibitory concentrations (MIC) in Table 3. Generally all samples showed some level of activity except compounds **4** and **7**. It is also evident from the table that there is some correlation between antimicrobial activity and the lethality bioassay, with piliostigmol, **1**, showing the highest activity against *E. coli* (MIC = 2.57 μg/ml; 0.006 μmol) which is about three times that of amoxicillin, whereas **4** and **7** showed the least activity, there being no observable activity for **7** against Gram-negative bacteria and the fungus *Aspergillus niger*.

In general, flavonoids and oxychromones are known to exhibit a wide range of activities including anti inflammatory, antithrombotic, antiviral and hepatoprotection which, in some measure, may be due to their ability to scavenge free-radicals (Aderogba et al., 2006; Akdemir et al., 2001; Saija et al., 1995). Some specific flavonoids have been reported to be potent inhibitors of indole-3-acetic acid oxidase activity (Ferrer et al., 1992). Among quercetins some have been shown to exhibit strong lipid peroxidation inhibitory effects and cytotoxicity (Cos et al., 2001) when tested against oral microorganisms. Thus, the present findings on the antimicrobial activities of the C-methylated flavonols and the oxychromonol isolated from *P. reticulatum* (Ibewuike et al., 1997) lend support to the well founded effectiveness of flavonoids and closely related structures against microbial infections, and hence provide justification for the usage of this plant in folk medicine for the treatment of viral infections, oral sores and inflammations (Iinuma et al., 1994; Kirtikar et al., 1993; Yusuf et al., 1994; Liu et al., 1990).

3. Materials and methods

3.1. Experimental

3.1.1. General experimental procedures

The IR spectra were recorded with a Mat. Galaxy 5000 series FT-IR spectrometer, while UV spectra were obtained from a Unicam UV 4-100 UV/Vis spectrophotometer. The MS were obtained using JEOL JMS-AX505HA double-focusing probe at 70 eV, while NMR spectra (both 1D and 2D) were obtained on a Bruker AMX-400 (at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) spectrometer in DMSO-d_6. Vacuum liquid chromatography (VLC) was carried out using Merck Silica gel 60 H, while gel filtration was performed using Sephadex LH-20 (Sigma). Preparative TLC was performed using Merck Silica gel 60 PF_{254} on glass plates (20 cm × 20 cm) and with a thickness of 0.5 mm. TLC was conducted on normal-phase Merck Silica gel 60 PF_{254} on precoated aluminium plates. Separated compounds on TLC and PTLC plates were visualized under UV light at (254 and 366 nm), and spraying of the plates where required was carried out using 2% vanillin in H_2SO_4 followed by heating at 110 °C for 2–5 min. Quercetin was used as TLC standard.

Plant material: Fresh leaves of *P. reticulatum* (Schum.) were collected in March 2005 at Ajebanbo Village, close to Ondo Town in the South Western part of Nigeria and authenticated by Dr. A. Ayoola of the Department of Biology, Obafemi Awolowo University, (ACE Campus, Ondo). A voucher specimen (specimen no.: ACEBH 6266) has been deposited at the College Herbarium. The leaves were dried, milled into a powder and stored in air-tight stoppered glassware prior to analysis. For the purposes of phytochemical screening, portions of the milled sample were soaked separately in three solvents, namely petroleum ether, dichloromethane and methanol, at room temperature (25 °C) for 24 h, and the extracts so derived were evaporated on a rotary evaporator at ⩽45 °C. Phytochemical screening for the detection of natural products such as tannins, phenolics, glycosides, saponins, flavonoids, alkaloids anthraquinones, steroids, essential oils and terpenes was performed according to the method of Wagner and Bladt (2001).

3.2. Extraction and isolation

Dried, milled plant material (2 kg) was sequentially extracted with *n*-hexane (b.p. 66–70 °C), ethyl acetate (b.p. 75–77 °C) and methanol (b.p. 64–65 °C) in a Soxhlet apparatus. The extracts were concentrated *in vacuo*. Vacuum liquid chromatography (VLC) of the

hexane extract (28 g) on silica gel was performed using an increasing gradient of petroleum ether (PE) and EtOAc and later on MeOH was also introduced. Preparative TLC with 5% EtOAc in toluene was carried out on the VLC fraction which had been eluted with 10% EtOAc in PE from VLC and this gave compound **2** (27.2 mg). Recrystallisation from ethanol of the VLC fraction eluted with 15% EtOAc in PE yielded **1** (13.3 mg). Compound **3** (10.5 mg) was recrystallised from the subsequent VLC fractions obtained with 20–25% EtOAc in PE.

The ethyl acetate extract (25 g) was fractionated by VLC over Silica gel 60H using PE–EtOAc and EtOAc–MeOH mixtures of increasing polarity. The fractions obtained were combined on the basis of their respective TLC profiles. The VLC fractions eluted with 20–25% EtOAc in petroleum ether were further subjected to column chromatography on Sephadex LH-20 eluting with 20% EtOAc in toluene yielding **4** (12.7 mg). The VLC fractions eluted with 30–35% EtOAc in PE, upon further chromatography on Sephadex, using PE:toluene:MeOH (2:5:1) gave more of **2** (5.4 mg) and **7** (7.5 mg). Compounds **5** (8.4 mg) and **6** (7.6 mg) were isolated from the VLC fractions eluted with 50% EtOAc in PE followed by chromatography on Sephadex eluting with a gradient of 5–10% MeOH in toluene to 100% MeOH. Final purification was achieved by preparative TLC with 30% EtOAc in PE.

The methanol extract (32.5 g) was fractionated using VLC on silica gel 60H eluting with mixtures of toluene and methanol of increasing polarity. Chromatography of the VLC fraction eluted with 5–10% MeOH in toluene on Sephadex using 20% MeOH in toluene yielded more of **4** (10.0 mg) while later VLC fractions (fraction eluted with 20–25% MeOH in toluene) yielded more of **6** (6.5 mg) on Sephadex using 20% MeOH in toluene.

3.2.1. (**1**) Piliostigmol

Creamy needle-like crystals, m.p. 220–224 °C.

UV λ_{max} nm (log ε): 230 (4.89), 286 (4.59); +NaOAc: 232, 287; +NaOH: 244, 288; +A1C1$_3$: 234, 252 (sh), 308, 351.

IR (ν cm^{-1}) pronounced peaks: 3435, 3212, 1658, 1606, 1566.

EIMS: molecular ion peak $(M)^+$ *m/z* 330.0681 (calculated molecular formula, $C_{17}H_{14}O_7$).

^{1}H NMR: δ 2.01 (3H, s, Me-6), 3.90 (3H, s, OMe-7), 6.72 (1H, s, H-8), 6.85 (2H, d, *J* = 8.8 Hz, H-3′, 5′), 7.19 (2H, d, *J* = 8.8 Hz, H-2′, 6′), 9.78 (1H, s, 4′-OH), 11.65 (1H, s, 3-OH), 12.95 (1H, s, 5-OH).

^{13}C NMR: see Table 1.

3.2.2. (**2**) 6,8-Di-C-methylquercetin-3,3′,7-trimethyl ether

Yellow waxy powder, m.p. 191–193 °C.

UV λ_{max} nm (log ε): 257 (4.64), 355 (4.62); +NaOAc: 270, 365; +NaOH: 276, 342, 410. +AlCl$_3$: 279, 440.

IR (ν cm^{-1}) pronounced peaks: 3401, 2932, 1653, 1612, 1556.

EIMS: molecular ion peak $(M)^+$ *m/z* 372.1103 (calculated molecular formula $C_{20}H_{20}O_7$).

^{1}H NMR: δ 2.12 (3H, s, Me-6), 2.36 (3H, s, Me-8) 3.78 (3H, s, OMe-3), 3.97 (3H, s, OMe-3′), 4.03 (3H, s, OMe-7); 6.91 (1H, d, *J* = 8.5 Hz, H-5′), 7.60 (1H, dd, *J* = 2.3, 8.4 Hz, H-6′), 7.70 (1H, d, *J* = 2.3 Hz, H-2′), 10.20 (1H, s, 4′-OH), 12.92 (1H, s, 5-OH).

^{13}C NMR: see Table 1.

3.2.3. (**3**) 6,8-Di-C-methylquercetin-3,3′-dimethyl ether

Yellow fluffy powder, m.p. 265–267°C.

UV λ_{max} nm (log ε): 262 (4.62), 354 (4.60); +NaOAc: 260, 355; +NaOH: 270, 405; +A1C1$_3$: 277, 435.

IR (ν cm^{-1}) pronounced peaks: 3429, 1652, 1613, 1568, 1556.

EIMS: molecular ion peak $(M)^+$ *m/z* 360.0954 (calculated molecular formula $C_{19}H_{18}O_7$).

^{1}H NMR: δ 2.11 (3H, s, Me-6), 2.41 (3H, s, Me-8); 3.89 (3H, s, OMe-3), 3.98 (3H, s, OMe-3′), 7.03 (1H, d, *J* = 8.6 Hz, H-5′), 7.62 (1H, dd, *J* = 2.3, 8.6 Hz, H-6′), 7.75 (1H, d, *J* = 2.3 Hz, H-2′), 10.23 (1H, s, 4′-OH), 12.95 (1H, s, 7-OH).13.05 (1H, s, 5-OH).

^{13}C NMR: see Table 1.

3.2.4. (**4**) 3′,6,8,-Tri-C-methylquercetin-3,7-dimethyl ether

Yellow amorphous powder, m.p. 243–246 °C.

UV λ_{max} nm (log ε): 263 (4.65), 363 (4.62); +NaOAc: 262, 367; +NaOH: 267, 412; +A1C1$_3$: 281, 447.

IR (ν cm^{-1}) pronounced peaks: 3356, 1642, 1610, 1578, 1546.

EIMS: molecular ion peak $(M)^+$ *m/z* 356.3821 (calculated molecular formula $C_{20}H_{20}O_6$).

^{1}H NMR: δ 2.16 (3H, s, Me-6), 2.29 (3H, s, Me-3′), 2.42 (3H, s, Me-8), 3.84 (3H, s, OMe-3), 3.92 (3H, s, OMe-7), 7.02 (1H, d, *J* = 8.6 Hz, H-5′), 7.62 (1H, dd, *J* = 2.3, 8.6 Hz, H-6′), 7.74 (1H, d, *J* = 2.3 Hz, H-2′), 10.13 (1H, s, 4′-OH), 13.02 (1H, s, 5-OH).

^{13}C NMR: see Table 1.

3.2.5. (**5**) 6-C-methylquercetin-3-methyl ether

Yellow powder, m.p. 261–263 °C.

UV and IR data were in agreement with that of Harborne et al., 1994.

EIMS: molecular ion peak $(M)^+$ *m/z* 330.2642 (calculated molecular formula $C_{17}H_{14}O_7$).

^{1}H MMR: δ 2.17 (3H, s, M-6), 3.92 (3H, s, OMe-3), 6.66 (1H, s, H-8), 7.10 (1H, d, *J* = 8.6 Hz, H-5′), 7.64 (1H, dd, *J* = 2.3, 8.6 Hz, H-6′), 7.76 (1H, d, *J* = 2.3 Hz, H-2′), 10.23 (1H, s, 4′-OH), 13.10 (1H, s, 5-OH).

3.2.6. (**6**) 6,8-Di-C-methylkaempferol-3-methyl ether

Yellow waxy amorphous solid, m.p. 250–253 °C.

UV and IR data were in agreement with that of Voirin, 1983.

EIMS: molecular ion peak $(M)^+$ *m/z* 329.2112 (calculated molecular formula $C_{18}H_{16}O_6$).

^{1}H NMR: δ 2.15 (3H, s, Me-6); 2.35 (3H, s, Me-8); 3.88 (3H, s, OMe-3); 8.05 (2H, d, *J* = 9 Hz, H2′, 6′); 7.10 (2H, d, *J* = 9 Hz, H-3′,5′); 10.11 (1H, s, 4′-OH), 13.00 (1H, s, 5-OH).

3.2.7. (**7**) 6-C-methylquercetin-3,3′,7-trimethyl ether

Yellow needle-like crystals, m.p. 184–187 °C.

UV and IR data were in agreement with that of Rabesa and Voirin, 1985.

EIMS: molecular ion peak $(M)^+$ *m/z* 358.4133 (calculated molecular formula $C_{19}H_{18}O_7$).

^{1}H NMR: δ 2.08 (3H, s, Me-6); 3.91 (3H, s, OMe-3); 3.98 (3H, s, OMe-3′); 4.01 (3H, s, OMe-7); 6.90 (1H, s, H-8); 7.07 (1H, d, *J* = 8.6 Hz, H-5′); 7.72 (1H, dd, *J* = 2.3, 8.6 Hz, H-6′); 7.77 (1H, d, *J* = 2.3 Hz, H-2′). 10.10 (1H, s, 4′-OH), 12.95 (1H, s, 5-OH).

3.3. The brine shrimp lethality biossay

The brine shrimp lethality biossay was carried out according to the method described by Meyer (Meyer et al., 1982). Brine shrimp eggs (*Artemia salina* Leach) were hatched in artificial sea water (35 g/l of sea salt, Sigma Chemicals) using a large soap case as an artificially partitioned dam and they were incubated at room temperature for 48 h. With the help of a light source, the larvae (nauplii) were attracted to one side of the vessel and easily collected for the assay. The three crude extracts, and the seven isolates **1–7** were dissolved in dimethyl sulfoxide (DMSO) at a maximum concentration not exceeding 0.05%, and then diluted with sea water for testing at the final concentrations of 10, 100 and 1000 μg/ml. Each test was conducted in triplicate. Ten nauplii were used for each test. Nauplii were counted under a magnifying glass after 24 h of incubation and maintaining the vials under illumination. The controls were prepared in the same manner except that the test samples were omitted. The number of dead nauplii was

recorded and it was used for calculating the LC_{50} by the Finney Probit analysis program. LC_{50} values greater than 1000 ppm or close to this value were considered inactive.

3.4. Antimicrobial screening

Antimicrobial analysis was carried out by using a newly developed microdilution titre techniques (Drummond and Waigh, 2000; Rahman and Gray, 2005) in which 96 well plates were used for the testing against the two Gram-positive bacteria (*Staphylococcus aureus* NCTC 6571 and *Bacillus subtilis* NCTC 8236) and the two Gram-negative bacteria (*Escherchia coli* NCTC 10418 and *Proteus vulgaris* NCTC 4175). The two fungi used were *A. niger* ATCC 10578 and *Candida albicans* ATCC 10231.

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