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ISOLATION, STRUCTURE ELUCIDATION, AND BIOLOGICAL ACTIVITY OF FLAVONE 6-C-GLYCOSIDES FROM *Alliaria petiolata*

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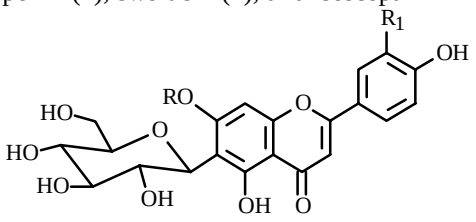
Preparative reversed-phase HPLC analysis of a methanol extract of the seeds of Alliaria petiolata afforded four flavone 6-C-glycosides: isoorientin, swertiajaponin, swertisin and isoscoparin-2″-β-D-glucopyranoside. The molecular structures were elucidated by UV, ESIMS and comprehensive 1D (^1H and ^{13}C) and 2D (gradient multiple quantum filtered ^1H-^1H COSY, ^1H-^{13}C HSQC and ^1H-^{13}C HMBC) NMR analyses. The antibacterial and free radical scavenging activity, and general toxicity of these compounds were assessed. While none of these glycosides showed any significant antibacterial activities at test concentrations, all these compounds showed prominent free radical scavenging activity (IC_{50} values: 1.25×10^{-2} to 7.69×10^{-3} mg/mL) in DPPH assay. In the brine shrimp lethality assay very low levels of general toxicity (LD_{50} >1.00 mg/mL) were displayed.

Key words: *Alliaria petiolata*, flavone 6-C-glycoside, antioxidant, NMR.

Alliaria petiolata (M. Bieb.) Cavara & Grande (family: Brassicaceae alt. Cruciferae), commonly known as "Garlic mustard," is a flowering weed native to a number of countries in Africa, temperate and tropical Asia, and Europe [1]. This plant is also widely distributed all over the U.K. It has been used as a food additive (flavoring agent) and also in folk medicine as an antiseptic, diuretic, expectorant, stimulant, and vermifuge, and to treat scurvy [1, 2]. Previous phytochemical studies on various parts of this plant yielded various cyano and amino compounds, fatty acids, ascorbic acid, arachidonic acid, cardenolides [2–4], and flavonoid C-glycosides [5–7]. We now report on the isolation, systematic structure determination by spectroscopic means, antibacterial and free radical scavenging activity, and general toxicity of four flavone 6-C-glycosides from the seeds of *A. petiolata.*

RP-HPLC analysis of the methanol extract of the seeds of *A. petiolata* afforded four flavone 6-C-glycosides which, on the basis of comprehensive spectroscopic analyses (e.g. UV, ESIMS, and 1D and 2D NMR), were characterized as isoorientin (**1**), swertiajaponin (**2**), swertisin (**3**), and isoscoparin-2″-*β*-D-glucopyranoside (**4**).



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1: R = H, R_1 = OH;
2: R = Me, R_1 = OH;
3: R = Me, R_1 = H

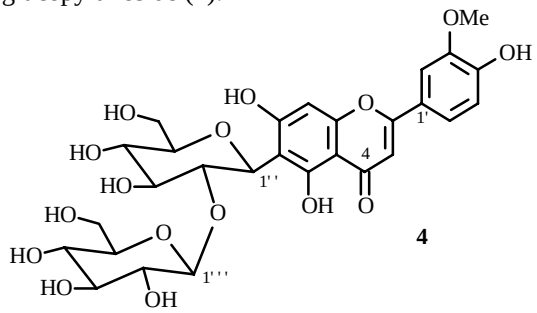

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TABLE 1. ^{1}H NMR Data (Coupling Constant J/Hz in Parentheses) of Compounds **1-4**

Carbon	Chemical shift δ in ppm				Carbon	Chemical shift δ in ppm			
	1	**2**	**3**	**4**		**1**	**2**	**3**	**4**
3	6.43 s	6.57 s	6.54 s	6.62 s	4″	3.50[a]	3.51[a]	3.51[a]	3.50[a]
7-OMe	-	3.90 s	3.90 s	-	5″	3.40[a]	3.41[a]	3.41[a]	3.38[a]
8	6.38 s	6.69 s	6.60 s	6.49 s	6″	3.71 dd (6.0, 11.8)	3.66 dd (6.0, 11.9)	3.72[a]	3.72[a]
2′	7.27[a]	7.38[a]	7.78 d (8.4)	7.43[a]		3.84 dd (6.8, 11.8)	3.83 dd (6.8, 11.9)	3.90[a]	3.88[a]
3′	-	-	6.87 d (8.4)	-	1‴	-	-	-	4.38 d (8.2)
3′-OMe	-	-	-	3.88 s	2‴	-	-	-	3.10[a]
5′	6.82 d (8.6)	6.86 d (8.6)	6.87 d (8.4)	6.90 d (8.6)	3‴	-	-	-	3.25[a]
6′	7.27[a]	7.38[a]	7.78 d (8.4)	7.43[a]	4‴	-	-	-	3.20[a]
1″	4.82[b]	4.88[b]	4.86[b]	4.94[b]	5‴	-	-	-	2.98[a]
2″	4.13 t (8.8)	4.14 t (8.8)	4.13 t (8.8)	4.58 bt	6‴	-	-	-	3.38[a]
3″	3.52[a]	3.56[a]	3.56[a]	3.60[a]					3.40[a]

Spectra obtained in CD_3OD; [a]Masked by water peak, confirmed from ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC experiments; [b]Overlapped peaks, confirmed from ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC.

TABLE 2. ^{13}C NMR Data of Compounds **1-4**

Carbon	Chemical shift δ in ppm				Carbon	Chemical shift δ in ppm			
	1	**2**	**3**	**4**		**1**	**2**	**3**	**4**
2	164.9	164.8	164.8	164.9	5′	115.6	115.1	115.2	115.2
3	102.6	102.5	102.5	102.6	6′	119.1	119.5	129.2	120.1
4	182.7	182.7	182.7	182.7	1″	74.0	73.5	73.5	71.5
5	160.7	160.8	160.8	160.7	2″	71.4	70.9	71.2	80.4
6	107.8	108.4	108.2	107.0	3″	78.9	78.2	79.5	78.5
7	163.6	163.4	163.8	163.6	4″	70.6	70.0	70.5	70.5
7-OMe	-	56.2	55.6	-	5″	81.4	80.6	81.0	81.0
8	94.0	90.0	90.0	93.2	6″	61.7	61.8	61.7	61.8
9	157.4	157.6	157.6	157.4	1‴	-	-	-	105.1
10	103.9	104.0	103.7	103.6	2‴	-	-	-	74.3
1′	122.3	122.1	122.0	122.2	3‴	-	-	-	76.3
2′	112.9	112.9	129.2	110.0	4‴	-	-	-	69.9
3′	145.7	145.2	115.2	147.5	5‴	-	-	-	76.6
3′-OMe	-	-	-	56.0	6‴	-	-	-	61.2
4′	149.8	149.0	161.0	150.0					

Spectra obtained in CD_3OD.

All four compounds (**1–4**) displayed characteristic UV absorption maxima for flavone skeleton [8]. The ^{1}H NMR (Table 1) and ^{13}C NMR data (Table 2) for these compounds also confirmed the presence of flavone nucleus in these molecules [8]. In the UV spectrum of **1**, the bathochromic shift of band I in presence of MeOH (without any decrease in intensity) and its stability after five minutes indicated the presence of 4′-hydroxy and absence of any of the following trihydroxy systems: 5, 6, 7 or 5, 7, 8 or 3′, 4′, 5′. The bathochromic shift of band I with $AlCl_3$ and its stability in the presence of HCl was due to the presence of free 5-hydroxy group. Similarly, while the bathochromic shift of band II with NaOAc was related to free 7-hydroxy group, that

of band I with $NaOAc/H_3BO_3$ was owing to the presence of *ortho*-dihydroxy in ring B. An ESIMS mass spectrum revealed $[M+H]^+$ (positive ion mode) ion peak at *m/z* 449, suggesting M_r = 448 and solving for $C_{21}H_{20}O_{11}$. In the ^1H and ^{13}C NMR spectra (Tables 1 and 2), there were signals for a 6-substituted 5,7,3′,4′-tetrahydroxy flavone nucleus and also for a glucose moiety. The ^1H and ^{13}C chemical shift values were comparable to the published data for the C-glucoside, isoorientin (**1**) [9, 10].

The gradient multiple quantum filtered ^1H-^1H COSY spectrum of **1** revealed all possible ^1H-^1H couplings and helped to assign all proton resonances, especially those of the glucose moiety. The ^1H-^{13}C HSQC showed all ^1J direct ^1H-^{13}C correlations and thus confirmed the assignment of all signals arising from the methine and methylene groups. Among all ^1H-^{13}C long-range (^2J and ^3J) correlations observed in the ^1H-^{13}C HMBC spectrum, the most important was the correlation from the glucose anomeric proton (H-1″, δ 4.82), ^2J to C-6 (δ 107.8) and ^3J to C-5 (δ 160.7) and C-7 (δ 163.6) which confirmed the C-glucosidation at C-6.

The UV spectra of **2** showed similar absorption patterns and shifts as observed in **1**. The only exception was the absence of a shift of band II with NaOAc which indicated the absence of free hydroxyl group at C-7. An ESIMS mass spectrum revealed $[M+H]^+$ (positive ion mode) ion peak at *m/z* 463, suggesting M_r = 462 and solving for $C_{22}H_{22}O_{11}$. The ^1H and ^{13}C NMR data (Tables 1 and 2) of **2** were similar to those of **1**, with the exception that the presence of a methoxy (instead of a hydroxy) was evident from the signals at δ_H 3.90 and δ_C 56.2. On the basis of the UV absorption data, this methoxy group could be placed at C-7. However, more conclusive evidence in support of this placement was present in the ^1H-^{13}C HMBC spectrum where a ^3J correlation was observed from the protons of methoxy group to C-7 (δ 163.4). The spectroscopic data were comparable to those published for swertiajaponin (**2**) [11, 12]. To our knowledge, the unambiguous and complete assignment of all proton and carbon chemical shifts for **2**, on the basis of the gradient multiple quantum filtered ^1H-^1H COSY, ^1H-^{13}C HSQC and ^1H-^{13}C HMBC experiments, is presented here for the first time (Tables 1 and 2).

In the UV spectrum of **3**, the bathochromic shift of band I in presence of MeOH (without a decrease in intensity) and its stability after five minutes was related to the presence of a free 4′-hydroxyl group and absence of any of the following trihydroxy systems: 5, 6, 7 or 5, 7, 8 or 3′, 4′, 5′. The bathochromic shift of band I with $AlCl_3$ and its stability in the presence of HCl was due to the presence of a free 5-hydroxyl group. Absence of bathochromic shift of band II with NaOAc was owing to the lack of a free hydroxyl group at C-7. Absence of any bathochromic shift of band I with $NaOAc/H_3BO_3$ suggested that there was no *ortho*-dihydroxy system in ring B. An ESIMS mass spectrum revealed $[M+H]^+$ (positive ion mode) ion peak at *m/z* 447, suggesting M_r = 446 and solving for $C_{22}H_{22}O_{10}$. In addition to the signals owing to a 6-substituted-5,7,4′-trioxygenated flavone nucleus, the ^1H and ^{13}C NMR spectra showed signals for a *β*-D-glucosyl unit and a methoxy group (Tables 1 and 2).

Considering the UV results, the methoxy group could be placed at C-7. However, the confirmation of the position of this methoxy group was obtained from a ^3J correlation from the methoxy signal (δ_H 3.90) to C-7 (δ_C 163.8) observed in the ^1H-^{13}C HMBC spectrum of **3**. Among the other ^1H-^{13}C long-range (^2J and ^3J) correlations observed in the ^1H-^{13}C HMBC spectrum, the most significant was the correlation from the glucose anomeric proton (H-1″, δ 4.86), ^2J to C-6 (δ 108.2) and ^3J to C-5 (δ 160.8) and C-7 (δ 163.8) which confirmed the C-glucosidation at C-6. The gradient multiple quantum filtered ^1H-^1H COSY spectrum of **3** revealed all possible ^1H-^1H couplings and helped assigning the all proton resonances, especially of the glucose moiety. The ^1H-^{13}C HSQC showed all ^1J direct ^1H-^{13}C correlations and thus confirmed the assignment of all signals arising from the methine and methylene groups. The spectroscopic data were comparable to those published for swertisin (**3**) [13, 14].

In the UV spectrum of **4**, the bathochromic shift of band I in presence of MeOH (without a decrease in intensity) and its stability after five minutes were related to a free 4′-hydroxy group and absence of any of the following trihydroxy systems: 5, 6, 7 or 5, 7, 8 or 3′, 4′, 5′. While the bathochromic shift of band I with $AlCl_3$ and its stability in the presence of HCl were owing to the presence of a free hydroxyl group at C-5, the shift of band II with NaOAc was related to a free 7-hydroxyl. The absence of bathochromic shift of band I with $NaOAc/ H_3BO_3$ indicated that there was no *ortho*-dihydroxy system in ring B. An ESIMS mass spectrum revealed $[M+H]^+$ (positive ion mode) ion peak at *m/z* 625, suggesting M_r = 624 and solving for $C_{28}H_{32}O_{16}$. In addition to the signals owing to an isoorientin (**1**) nucleus, the ^1H and ^{13}C NMR spectra showed signals for another *β*-D-glucosyl unit and a methoxy group (Tables 1 and 2). Considering the UV results, the methoxy group could be placed at C-3′. However, the confirmation of the position of this methoxy group was achieved from a ^3J correlation from the methoxy signal (δ_H 3.88) to C-3′ (δ_C 147.5) observed in the ^1H-^{13}C HMBC spectrum of **4**.

TABLE 3. ^{1}H-^{13}C HMBC Key Long-range Correlations (^{2}J and ^{3}J) in **4**

Proton signals	^{1}H-^{13}C long-range correlations		Proton signals	^{1}H-^{13}C long-range correlations	
	^{2}J	^{3}J		^{2}J	^{3}J
H-3	C-4	C-1′, C-10	H-4″	C-3″, C-5″	C-6″
H-8	C-7, C-9	C-6, C-10	H-5″	C-6″	
H-2′	C-3′	C-2, C-4′, C-6′	H-6″		C-4″
3′-OMe		C-3′	H-1‴		C-2″, C-3‴, C-5‴
H-5′	C-4′, C-6′	C-1′, C-3′	H-2‴	C-1‴, C-3‴	
H-6′	C-1′, C-5′	C-2′, C-4′	H-3‴	C-2‴	C-1‴
H-1″	C-6	C-5, C-7, C-3″, C-5″	H-4‴	C-3‴, C-5‴	C-6‴
H-2″	C-1″	C-1‴	H-5‴	C-6‴	
H-3″		C-1″	H-6‴		C-4″

The significant deshielding of the ^{1}H and ^{13}C signals (δ_H 4.58 and δ_C 80.4) associated with C-2″ of the glucose unit of isoorientin (**1**) indicated further glucosylation at C-2″. Thus the extra glucose unit could be placed at C-2″. The β 1→2 link between two sugars was confirmed from a ^{3}J correlation from the anomeric proton (δ_H 4.38) of the second glucose unit to C-2″ observed in the ^{1}H-^{13}C HMBC spectrum (Table 3). The gradient multiple quantum filtered ^{1}H-^{1}H COSY spectrum of **4** revealed all possible ^{1}H-^{1}H couplings and helped assigning the all proton resonances, especially of the glucose moieties. The ^{1}H-^{13}C HSQC showed all ^{1}J direct ^{1}H-^{13}C correlations and thus confirmed the assignment of all signals arising from the methine and methylene groups. The spectroscopic data were comparable to those published for isoscoparin 2″-O-β-D-glucopyranoside (**4**) [15]. To our knowledge, the unambiguous and complete assignment of all proton and carbon chemical shifts for **4**, on the basis of 2D NMR experiments, is presented here for the first time (Tables 1 and 2).

To our knowledge, this is the first report on the occurrence of these flavone 6-C-glycosides (**1–4**) in the seeds of *Alliaria petiolata*. However, only two other C-glycosides, i.e., isovitexin 6″-O-β-D-glucopyranoside and 6″-O-[4-hydroxy-3,5-dimethoxy-*E*-cinnamoyl-(-6)-β-D-glucopyranosyl]-isovitexin, have previously been reported from the aerial parts of this plant [6, 7]. Among the glycosides reported in this paper, while isoorientin (**1**) is one of the most common flavone 6-C-glycosides found in the plant kingdom, compounds **2–4** are distributed only in a few plant families [7]. Swertiajaponin (**2**) is distributed only in the genera *Achillea* (Family: Asteraceae), *Cephalaria* (Family: Dipsacaceae) *Cumis* (Family: Cucurbitaceae), *Passiflora* (Family: Passifloraceae), *Phragmites* (Family: Poaceae), and *Swertia* (Family: Gentianaceae) [7, 11, 12]. Swertisin (**3**) has also been reported from a number of genera, e.g. *Swertia*, *Gaillardia*, *Gentiana*, *Enicostemma*, *Iris*, *Ziziphus*, *etc.* [7, 13, 14]. Compound **4** was previously found in the aerial parts of *Passiflora incarnata* [15] and *Oryza sativa* [16], but identified merely on the basis of UV and ^{1}H NMR data.

All four flavone 6-C-glycosides (**1–4**) showed prominent free radical scavenging activity (antioxidant activity) in the DPPH assay [17, 18]. The IC_{50} of **1–4** were found to be 3.92×10^{-3}, 3.95×10^{-3}, 7.69×10^{-3} and 1.25×10^{-2}, mg/mL, respectively, compared to 2.88×10^{-5} mg/mL for quercetin and 3.08×10^{-3} mg/mL for trolox, two well-known antioxidants. The antioxidant activity of **1**–**4**, like other natural phenolic antioxidants, e.g., flavonoids [19–22], is a consequence of the presence of the phenolic moieties in the structures. The antioxidant activity of phenolic natural products is predominantly owing to their redox properties, i.e., the ability to act as reducing agents, hydrogen donors, and singlet oxygen quenchers, and to some extent, could also be due to their metal chelation potential [23].

The brine shrimp lethality assay, which has been proven to be an effective and rapid assay method to screen compounds for potential cytotoxic activity [24], was used to determine the general toxicity of these flavone C-glycosides (**1–4**). From Probit analysis [25], the LD_{50} values of **1–4** were found to be > 1000 µg/mL, whereas that of the control podophyllotoxin, a well known cytotoxic lignan, was 2.79 µg/mL. The high LD_{50} values of these compounds indicated very low general toxicity, which could be useful if these compounds were to be treated as safe natural antioxidants.

None of these compounds **1–4** showed any antibacterial activity against the test organisms at test concentrations with the exception that **4** inhibited the growth of *Citrobacter ferundii* (MIC = 0.5 mg/mL).

EXPERIMENTAL

General. UV spectra were obtained in MeOH using a Hewlett-Packard 8453 UV-vis spectrometer. NMR spectra were recorded in CD_3OD on a Varian Unity INOVA 400 MHz NMR Spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C) using residual solvent peak as internal standard. CIMS and FABMS (positive ion mode) analyses were performed, respectively, on a Quattro II triple quadrupole instrument and Finnigan MAT95 spectrometer. HPLC separation was performed in a Dionex prep-HPLC System coupled with Gynkotek GINA50 autosampler and Dionex UVD340S Photo-Diode-Array detector. A Luna C_{18} preparative HPLC column (10 m, 250 mm × 21.2 mm) was used. Sep-Pak Vac 35 cc (10 g) C_{18} cartridge (Waters) was used for pre-HPLC fractionation.

Plant Material. Seeds of *A. petiolata* (M. Bieb.) Cavara & Grande (catalogue No: 15051) were purchased from B & T World Seeds sarl, Paguigan, 334210 Olonzac, France. A voucher specimen (PH500001) representing this purchase has been generated in the herbarium of Plant and Soil Science Department, University of Aberdeen, Scotland (ABD).

Extraction, Isolation, and Structure Elucidation. Ground seeds (100.0 g) of *A. petiolata* were Soxhlet-extracted successively with *n*-hexane, dichloromethane, and MeOH. All these extracts were separately concentrated using a rotary evaporator at a maximum temperature of 45°C. The MeOH extract was fractionated on a Sep-Pak, using 30, 60, 80 and 100% MeOH-water mixture (150 ml each) as eluent. Preparative RP-HPLC (gradient elution, 30–100% MeOH in water in 50 min, 20 ml/min.) of the Sep-Pak fraction (30% MeOH in water) yielded compounds **1–4** (95.2, 17.3, 18.5 and 4.8 mg, respectively). Structures of **1–4** were determined conclusively by UV, IR, ESIMS and extensive 1D (^1H and ^{13}C) and 2D (gradient multiple quantum filtered ^1H-^1H COSY, ^1H-^{13}C HSQC and ^1H-^{13}C HMBC) NMR analyses.

Isoorientin (1). Yellow amorphous solid. UV (MeOH, λ_{max}, nm): 257 (sh), 271, 348; + NaOMe: 270, 278 (sh), 327, 405; +$AlCl_3$: 277, 298 (sh), 344 (sh), 422; +$AlCl_3$ + HCl: 279, 292 (sh), 361 (sh), 380; + NaOAc: 269, 315, 404; +NaOAc + H_3BO_3: 265, 375, 430; ^1H- and ^{13}C NMR (Tables 1 and 2, refs: 9, 10). ESIMS (positive ion mode) *m/z* 449 $[M+H]^+$, 471 $[M+Na]^+$.

Swertiajaponin (2). Yellow amorphous solid. UV (MeOH, λ_{max}, nm): 255 (sh), 271, 348; + NaOMe: 268, 327 (sh), 405; +$AlCl_3$: 277, 294 (sh), 330 (sh), 425; +$AlCl_3$ + HCl: 259, 278, 292 (sh), 362, 378; + NaOAc: 268, 297 (sh), 407; +NaOAc + H_3BO_3: 265, 290 (sh), 375, 428 (sh); ^1H- and ^{13}C NMR (Tables 1 and 2, refs: 11, 12). ESIMS (positive ion mode) *m/z* 463 $[M+H]^+$, 485 $[M+Na]^+$.

Swertisin (3). Yellow amorphous solid. UV (MeOH, λ_{max}, nm): 255 (sh), 272, 338; + NaOMe: 270, 280 (sh), 394; +$AlCl_3$: 282, 298 (sh), 352 (sh), 380; +$AlCl_3$ + HCl: 280, 295, 349 (sh), 378; + NaOAc: 270, 293; +NaOAc + H_3BO_3: 271, 342; ^1H- and ^{13}C NMR (Tables 1 and 2, ref: 13, 14). ESIMS (positive ion mode) *m/z* 447 $[M+H]^+$, 469 $[M+Na]^+$.

Isoscoparin-2″-β-D-glucopyranoside (4). Yellow gum. UV (MeOH, λ_{max}, nm): 272, 344; + NaOMe: 269, 345 (sh), 409; +$AlCl_3$: 279, 293 (sh), 360 (sh), 392; +$AlCl_3$ + HCl: 281, 292 (sh), 354 (sh), 385; + NaOAc: 279, 318 (sh), 405; +NaOAc + H_3BO_3: 273, 351, 402 (sh); ^1H- and ^{13}C NMR (Tables 1 and 2, ref: 15). ESIMS (positive ion mode) *m/z* 625 $[M+H]^+$, 647 $[M+Na]^+$.

Free Radical Scavenging Activity (DPPH Assay): 2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. Quercetin was obtained from Avocado Research Chemicals Ltd, Shore road, Heysham, Lancs. The method used by Takao et al. [17] was adopted with suitable modifications to our particular circumstance [18]. DPPH (4 mg) was dissolved in MeOH (50 mL) to obtain a concentration of 80 μg/mL.

Qualitative assay: Test compounds **1–4** were applied on a TLC plate and sprayed with DPPH solution using an atomizer. It was allowed to develop for 30 min. The color changes (purple on white) were noted.

Quantitative assay: Compounds **1–4** were dissolved in MeOH to obtain a concentration of 0.5 mg/mL. Dilutions were made to obtain concentrations of 5×10^{-2}, 5×10^{-3}, 5×10^{-4}, 5×10^{-5}, 5×10^{-6}, 5×10^{-7}, 5×10^{-8}, 5×10^{-9}, 5×10^{-10} mg/mL. Diluted solutions (1.00 mL each) were mixed with DPPH (1.00 mL) and allowed to stand for half an hour for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the standards (quercetin and trolox).

Brine Shrimp Lethality Assay. Brine shrimp eggs were purchased from Water Life, Middlesex, UK. The bioassay was conducted following the procedure published previously [18, 24]. LD_{50} were determined from the 24 hour counts using the probit analysis method [25]. Percentage mortalities were adjusted relative to the natural mortality rate of the control, following Abbott's formula P = (Pi–C)/(1–C), where P denotes the observed nonzero mortality rate and C represents the mortality rate of the control.

Antibacterial Assay. Antibacterial activity of flavone C-glycosides **1–4** was assessed against 10 strains of Gram-positive and Gram-negative bacteria including *Bacillus subtilis* (NCIMB 7801), *Citrobacter freundii* (NCTC 9750), *Escherichia coli* (NCIMB 8110), *Escherichia coli* (NCIMB 4174), *Lactobacillus plantarum* (NCIMB 6376), *Proteus mirabilis* (NCIMB 60), *Pseudomonas aeruginosa* (NCTC 6750), *Salmonella goldcoast* (NCTC 13175), *Staphylococcus aureus* (NCTC 10788), and *Staphylococcus aureus* (MRSA) (NCTC 11940), using the 96-well micro-plate-based broth dilution method, also known as the checkerboard assay [18, 26–28]. Isosensitized nutrient broth was obtained from Oxoid, Basingstoke, Hampshire, England. Microtitre plates were from Sero-wel, Bibby sterilin, Stone, Staffs, UK. Eppendorf pipettes were purchased from Netheter-hinz-Gmbh, 22331, Hamburg, Germany. Bacterial suspension (20 μL) in double strength nutrient broth at a concentration of 5×10^5 CFU/mL was used. Test compounds (**1–4**) were dissolved in DMSO to obtain the stock concentration 1 mg/mL. Ciprofloxacin, a well known broad-spectrum antibiotic, was used as positive control. The minimum inhibitory concentration (MIC) was determined for each compound and compared with that of ciprofloxacin.

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