Antimicrobial and Antioxidant Isoflavonoid Derivatives from the Roots of *Amphimas pterocarpoides*

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A new bis-isoflavone derivative, named amphiisoflavone (**1**), was isolated from the roots of *Amphimas pterocarpoides*, together with three known isoflavones, namely 8-methoxyisoformononetin (**2**), 6-methoxyisoformononetin (**3**) and isoformononetin (**4**). Chemical transformations carried out on compound **3** led to two new derivatives, 4′-acetoxy-6,7-dimethylisoflavone (**3a**) and 4′-*O*-prenyl-6,7-dimethylisoflavone (**3p**), along with a known compound, 4′,6,7-trimethoxyisoflavone (**3m**). The structures of all compounds were elucidated from spectroscopic evidence, in particular by 1D and 2D NMR spectroscopic methods. These compounds were evaluated for their antioxidant properties and for their antimicrobial activities against a wide range of bacteria and fungi.

Key words: *Amphimas pterocarpoides*, Isoflavones, Chemical Transformations, Antimicrobial Properties, Antioxidant Activities

Introduction

The genus *Amphimas*, belonging to the family Leguminosae, occurs in tropical Africa and comprises only three species, namely *A. ferrugineus*, *A. klaineanus*, and *A. pterocarpoides* [1]. *Amphimas pterocarpoides* is a taller tree with heights up to 40 m of which different parts have been used in Cameroonian traditional medicine for the treatment of many diseases such as dysentery, aneamia, haematuria, dysmenorrhoea, blennorrhoea, schistosomiasis, cough, impotence, and mumps [2 – 4]. Furthermore, the stem bark of this plant is used as poison antidote while the inner bark is administered to prevent abortion [5, 6].

Inspite of these numerous usages, very few investigations have been carried out on *Amphimas pterocarpoides*, except those reported by Bevan *et al.* [7] and Tchoumtchoua *et al.* [8] who isolated respectively from the heartwood and from the stem bark of this plant, several isoflavonoids derivatives (afromosin, daidzein, irisolidone, *etc.*). The present work deals with the isolation, structural elucidation and chemical transformations of secondary metabolites from the roots of *Amphimas pterocarpoides* collected in Yaounde, Center region of Cameroon. Evaluation of the antimicrobial and antioxidant activities of these compounds is also reported.

Results and Discussion

Air-dried and powdered roots of *A. pterocarpoides* were extracted at room temperature with a mixture of CH_2Cl_2-MeOH (1 : 1). The residue obtained after evaporation of solvents was subjected to silica gel column chromatography eluted with a hexane-AcOEt mixture of increasing polarity to afford four compounds comprising a new bis-isoflavone **1** together with three known isoflavones, 8-methoxyisoformononetin (**2**), 6-methoxyisoformononetin (**3**) and isoformononetin (**4**). The structure of compound **1** was determined using spectroscopic data. The known metabolites were identified from their NMR and mass spectral data by comparison of these data and the physical constants with literature values [9 – 13].

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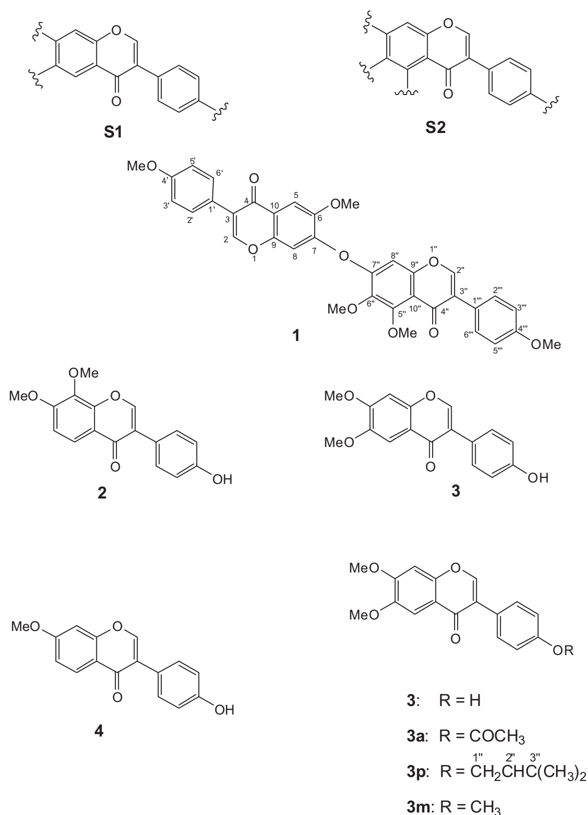

Table 1. ^{13}C NMR (75 MHz, [D$_6$]DMSO) data of compounds **1**, **3**, **3a**, and **3p**.

	δ_C (multiplicity)			
Position	**1**	**3**	**3a**	**3p**
2	151.8 (d)	152.9 (d)	154.4 (d)	153.5 (d)
3	124.2 (s)	122.6 (s)	123.5 (s)	123.8 (s)
4	174.7 (s)	174.2 (s)	174.9 (s)	174.3 (s)
5	105.3 (d)	104.7 (d)	113.7 (d)	114.6 (d)
6	147.5 (s)	152.7 (s)	150.5 (s)	153.8 (s)
7	153.1 (s)	147.0 (s)	144.9 (s)	148.1 (s)
8	103.1(d)	102.8 (d)	106.4 (d)	104.6 (d)
9	147.5 (s)	151.7 (s)	149.8 (s)	152.1 (s)
10	116.7 (s)	116.2 (s)	122.7 (s)	117.3 (s)
1′	123.1 (s)	124.4 (s)	124.4 (s)	124.8 (s)
2′,6′	130.6 (d)	130.0 (d)	130.6 (d)	130.5 (d)
3′,5′	114.0 (d)	113.6 (d)	114.2 (d)	114.1 (d)
4′	159.4 (s)	158.9 (s)	159.6 (s)	159.4 (s)
5″-OMe	62.1 (q)	–	–	–
6-OMe	61.3 (q)	55.7 (q)	55.6 (q)	55.6 (q)
6″-OMe	56.3 (q)	–	–	–
7-OMe	–	55.1 (q)	55.8 (q)	55.2 (q)
4′-OMe	55.6 (q)	–	–	–
4‴-OMe	55.6 (q)	–	–	–
4′-(COCH$_3$)	–	–	168.6 (s), 20.8 (q)	–
1″	–	–	–	62.2 (t)
2″	152.2 (d)	–	–	101.6 (d)
3″	124.9 (s)	–	–	138.9 (s)
4″	174.3 (s)	–	–	–
5″	153.4 (s)	–	–	–
6″	139.9 (s)	–	–	–
7″	156.6 (s)	–	–	–
8″	99.91 (d)	–	–	–
9″	139.9 (s)	–	–	–
10″	112.3 (s)	–	–	–
1‴	124.9 (s)	–	–	–
2‴, 6‴	130.7 (d)	–	–	–
3‴, 5‴	114.1 (d)	–	–	–
4‴	159.4 (s)	–	–	–
(*Z*)-Me	–	–	–	18.5 (q)
(*E*)-Me	–	–	–	25.9 (q)

Compound **1** was isolated as yellow needles, m. p. 190 – 192 °C. Its molecular formula $C_{35}H_{28}O_{10}$, corresponding to 22 double bond equivalents was deduced from positive HR-ESI-TOF-MS which showed the quasi molecular ion peak $[M+K]^+$ at $m/z = 627.1615$ (calcd. 627.1682 for $C_{35}H_{28}O_{10}K$).

The broad-band proton-decoupled ^{13}C NMR spectrum (75 MHz, [D$_6$]DMSO, Table 1) revealed the presence of 31 carbon signals instead of 35 as shown in the molecular formula, suggesting the presence of symmetry elements in **1**. These signals were sorted by DEPT 135 and HMQC techniques as five methyls, thirteen methines and seventeen quaternary carbon atoms among which there are two carbonyl groups at δ_C = 174.7 and 174.3 ppm.

Analysis of the ^{1}H NMR spectrum (300 MHz, [D$_6$]DMSO, Table 2) of compound **1** combined with the HMQC informations revealed a pair of one proton singlets at δ_H/δ_C = 8.33/151.8 and δ_H/δ_C = 8.19/152.2 ppm corresponding to two H-2 protons of the isoflavone skeleton [11, 14]. These data, combined with the molecular formula ($C_{35}H_{28}O_{10}$), suggested that compound **1** is a dimer containing two isoflavone moieties.

This assumption was further confirmed by the complementary information from the ^{1}H NMR spectrum (300 MHz, [D$_6$]DMSO, Table 2) which exhibited two AA′BB′ spin systems respectively at [δ_H = 7.45 (2H, d, J = 8.5 Hz) / δ_C = 130.6, H-2′, H-6′, δ_H = 7.52 (2H, d, J = 8.5 Hz) / δ_C = 130.7 ppm, H-2‴, H-6‴] and [δ_H = 6.98 (2H, d, J = 8.5 Hz) / δ_C = 114.0, H-3′, H-5′, δ_H = 7.00 ppm (2H, d, J = 8.5 Hz) / δ_C = 114.1, H-3‴, H-5‴], corresponding to two 1, 4-disubstituted benzene rings, attributed to the B ring protons of two isoflavone units [11]; a set of three one proton singlets

Table 2. ^{1}H NMR (300 MHz, [D_6]DMSO) data of compounds **1**, **3**, **3a**, and **3p**.

	δ_H (multiplicity, coupling constant *J* in Hz)			
Position	**1**	**3**	**3a**	**3p**
2	8.33 (s)	8.30 (s)	8.42 (s)	8.42 (s)
5	7.45 (s)	7.40 (s)	7.45 (s)	7.49 (s)
8	6.95 (s)	6.90 (s)	7.24 (s)	7.24 (s)
8′′	6.75 (s)	–	–	–
2′, 6′	7.45 (d, 8.5)	7.50 (d, 8.5)	7.53 (d, 8.5)	7.53 (d, 8.5)
2′′′, 6′′′	7.52 (d, 8.5)	–	–	–
3′,5′	6.98 (d, 8.5)	7.00 (d, 8.5)	7.01 (d, 8.5)	7.10 (d, 8.5)
3′′′,5′′′	7.00 (d, 8.5)	–	–	–
5′′-OMe	3.80 (s)	–	–	–
6-OMe	3.89 (s)	3.88 (s)	3.91 (s)	3.87 (s)
6′′-OMe	3.79 (s)	–	–	–
7-OMe	–	3.75 (s)	3.81 (s)	3.80 (s)
4′-OH	–	10.60 (s)	–	–
4′-OMe	3.80 (s)	–	–	–
4′′′-OMe	3.80 (s)	–	–	–
1′′	–	–	–	4.69 (d, 7.9)
2′′	8.19(s)	–	–	5.50 (t, 7.9)
(*Z*)-Me	–	–	–	1.76 (s)
(*E*)-Me	–	–	–	1.78 (s)
4′-$COCH_3$	–	–	2.34 (s)	–

at δ_H / δ_C = 7.45 / 105.3, δ_H / δ_C = 6.95 / 103.1 and δ_H / δ_C = 6.75 / 99.9 ppm assigned to three protons occupying the two A rings. The downfield signal at δ_H / δ_C = 7.45 / 105.3 ppm was assigned to the H-5 proton next to the C-4 carbonyl group while the two latter signals were attributed to two H-8 protons of two isoflavone monomers [11]. These spectroscopic data suggested the first ring A to be 1, 2, 4, 5 tetrasubstituted (sub-structure **S1**) and the second to be pentasubstituted (sub-structure **S2**).

The other substituents on the two isoflavone monomers were five methoxy groups which appeared as five close three proton singlets between δ_H = 3.89 and 3.79 ppm. For the monomer **S1**, the methoxy groups were located at positions C-4′ and C-6 according to NOESY correlations (Fig. 1) observed between the C-4′methoxy protons (δ_H / δ_C = 3.80 / 55.6) and protons H-3′ (δ_H / δ_C = 6.98 / 114.0) and H-5′ (δ_H / δ_C = 6.98 / 114.0) on one hand, and between the C-6 methoxy protons (δ_H / δ_C = 3.89 / 61.3) and H-5 (δ_H / δ_C = 7.45 / 105.3 ppm) on the other hand. Concerning the monomer **S2**, the locations of the methoxy groups at positions C-4′′′, C-5′′ and C-6′′ were equally supported by NOESY correlations between the C-4′′′ methoxy protons (δ_H / δ_C = 3.80 / 159.4) and protons H-3′′′ (δ_H / δ_C = 7.00 / 114.1) and H-5′′′ (δ_H / δ_C = 7.00 / 114.1), and between both C-5′′ and C-6′′ methoxy protons (δ_H / δ_C = 3.79 / 153.4 and δ_H / δ_C = 3.80 / 139.9 ppm). For each sub-structure, H-8 or H-8′′ showed no NOESY correlation with a methoxy group. Moreover, the C-4′ or C-4′′′ substitution of both isoflavone monomers was evidently due to the *para*-disubstituted nature of rings B as indicated by the ^{1}H NMR data.

It remained for us to establish the linkage site between the two isoflavone moieties. From the description presented above, the only positions remaining as free sites in the two monomer units were

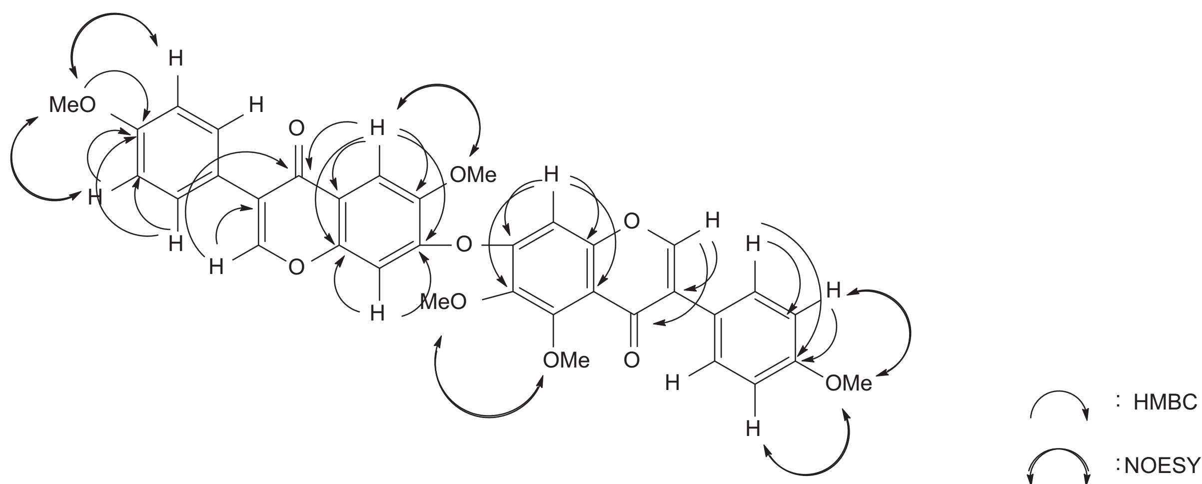

Fig. 1. HMBC and NOESY correlations of compound **1**.

position 7 on the first and position 7″ on the second monomer. Since in both monomers these positions were occupied by an oxygen atom, the linkage between the two sub-units is through C-7–O–C-7″. Therefore, compound **1** was determined as 4*H*-1-benzopyran-4-one, 7-[[6-methoxy-3-(4-methoxyphenyl)-4-oxo-4*H*-1-benzopyran-7-yl]oxy]-5,6-di-methoxy-3-(4-methoxyphenyl)-isoflavone, trivially named amphiisoflavone. The structure was further confirmed by HMBC correlations as shown in Fig. 1. Ultimate confirmation was due to its mass spectral fragmentation pattern.

Compounds **2** (8-methoxyisoformononetin), **3** (6-methoxyisoformononetin) and **4** (isoformononetin) were known compounds which were identified by comparison of their spectroscopic data with literature values [9–13]. Although compounds **2** and **3** were previously obtained from metabolism of daidzein and from microorganisms, respectively [9, 10], it is the first time that these compounds are reported from a plant, to the best of our knowledge.

In order to initiate a study of structure-activity relationship, acetylation, *O*-prenylation and methylation reactions were carried out on compound **3** obtained in significant amounts (300 mg), to yield **3a**, **3p** and **3m**, respectively. These derivatives were characterized by comparison of their NMR spectra with those of the parent compound **3**. The comparison of the ^{1}H NMR spectra of **3** and **3a** revealed not only the disappearance of the phenolic proton signal, but also the appearance of an additional three proton singlet at $\delta_H = 2.34$ ppm (3H, s) due to an acetyl group. Similar comparison of the ^{13}C spectra revealed two additional carbon atoms including one methyl at $\delta_C = 20.8$ (q) and one carbonyl carbon at $\delta_C = 168.6$ ppm (s). Regarding the prenylated derivative **3p**, its ^{1}H NMR spectrum showed, in addition to the disappearance of the phenolic proton signal, a set of four additional peaks at $\delta_H = 4.69$ (2H, d, $J = 7.9$ Hz), 5.50 (1H, t, $J = 7.9$ Hz), 1.76 (3H, s), and 1.78 ppm (3H, s) establishing the presence of an *O*-γ,γ-dimethylallyl moiety. This was confirmed by the ^{13}C signals observed at $\delta_C = 66.2$ (t), 101.6 (d), 138.9 (s), 18.5 (q), and 25.9 ppm (q). Comparison of the ^{1}H NMR spectra of **3** and the methylated derivative **3m** revealed a supplemental methoxy group in **3m** by a singlet appearing at $\delta_H = 3.81$ ppm. Amongst these derivatives, **3a** and **3p** are reported here for the first time whereas **3m** was already described [7, 9, 12].

The results of the antibacterial assays indicated that neither the extract nor the isolated compounds have good activities. However, better effects were obtained with the hemi-synthesized compounds (Table 3). The crude extract as well as compound **3a** showed good DPPH radical scavenging activity (IC_{50} of 63.59 μg mL^{-1} and IC_{50} of 70 μg mL^{-1}, respectively) (Table 4). However, all the studied samples showed a dose-dependent antioxidant activity (Fig. 2). When regarding the structure-activity relationship, it appears that, though compound **3** did not have any an-

Table 3. MIC values of the compounds and chloramphenicol on the tested microorganisms[a].

Samples	Microorganisms and MIC (μg mL^{-1})									
		E. aerogenes		*P. stuartii*	*K. pneumoniae*		*E. coli*	*P. aeruginosa*		*CA*
	EA27	ATCC 13048	CM64	NEA16	29916	KP55	AG102	PAO1	PA124	ATC9002
Crude extract										
	–	1024	–	–	1024	–	–	–	–	–
Isolated compounds										
1	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–
Hemisynthetic compounds										
3a	256	256	64	256	–	64	–	–	–	128
3m	–	256	256	256	256	256	256	128	–	128
3p	128	256	256	256	–	–	–	–	–	256
CHL	128	4	4	64	32	16	16	8	32	–

[a] (–) > 256 μg mL^{-1} for compounds and 1024 for the crude extract; CHL: chloramphenicol; microorganisms: *Providencia stuartii* (NAE16), *Pseudomonas aeruginosa* (PA01, PA124), *Klebsiella pneumoniae* (KP55), *Escherichia coli* (AG102), *Enterobacter aerogenes* (ATCC13048, EA27, CM64), and *Candida albicans* (CA).

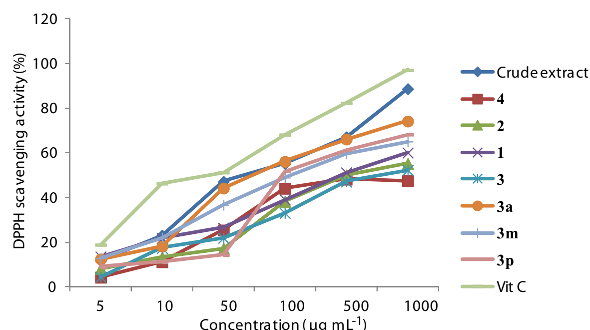

Fig. 2 (color online). DPPH scavenging activity of the studied compounds and vitamin C.

Table 4. IC_{50} values of samples as tested by the DPPH radical scavenging assay.

Tested samples	IC_{50} (μg mL^{-1})
Crude extract	63.59
1	> 1000
2	489.96
3	730.53
4	424.31
3a	70
3m	116.80
3p	96.59
Vitamin C	33.24

tibacterial activity and displayed poor antioxidant effect (IC_{50} of 730.53 μg mL^{-1}), the addition of either an acetyl (**3a**), methyl (**3m**), or prenyl (**3p**) group at the 4′-OH position induces an increase of both activities. In the antibacterial test, it was observed that compound **3m** showed a better spectrum of activity [active on 8 of the 10 (80 %) tested bacteria] than **3a** (active on 60 % of the tested bacteria) and **3p** (active on 50 % of the tested bacteria). It was also observed that compound **3a** displayed the lowest MIC values of 64 μg mL^{-1} against two of the studied bacteria (*E. aerogenes* CM64 and *K. pneumoniae* KP55). It can consequently be suggested that the methylation (**3m**) of compound **3** induces a significant increase of the antibacterial spectra meanwhile its acetylation (**3a**) induces a more selective and pronounced activity in certain cases. The 4′-*O*-prenylation induced lower effects than the corresponding acetylation and methylation. In the antioxidant test, it was observed that the 4′-*O*-acetylation (**3a**) induced a significantly higher effect (IC_{50} of 70 μg mL^{-1}) than prenylation (**3p**, IC_{50} of 96.56 μg mL^{-1}) and methylation (**3m**, IC_{50} of 116.80 μg mL^{-1}). It can therefore obviously been proposed that acetylation can be a better way to increase the biological activity of compound **3**. However, none of the observed effects were better than those of the studied reference drugs (chloramphenicol in antibacterial and vitamin C in antioxidant tests). Nonetheless, the antioxidant activity of **3a** was 2.11 fold lower than that of vitamin C, suggesting a possible use of this compound as antioxidant drug.

Experimental

General experimental procedures

Melting points were recorded on a Büchi apparatus and were not corrected. Column chromatography was performed on silica gel (Merck 70–230 and 230 – 400 mesh). Fractions were monitored by TLC using Merck pre-coated silica gel sheets (60 F_{254}), and spots were detected by ultraviolet radiation (254 or 366 nm) and by heating plates sprayed with 10 % H_2SO_4 solution. The mass spectra were measured on a Jeol MS Route instrument. 1D and 2D NMR spectra were recorded on a Bruker spectrometer operating at 300 (^{1}H) and 75 MHz (^{13}C). Chemical shifts (δ) are given in ppm with tetramethylsilane (TMS) as internal standard, and coupling constants (J) are in Hz.

Plant material

The roots of *Amphimas pterocarpoides* were collected in May 2011 at Yaoundé, Center region of Cameroon, and identified by Mr. Nana Victor (plant taxonomist) of National Herbarium Yaoundé, where a voucher specimen is deposited under n° 42198 HNC.

Extraction and isolation

Dried and powdered roots of *Amphimas pterocarpoides* (7.5 kg) were extracted with a mixture of CH_2Cl_2-MeOH (1 : 1) at room temperature for 48 h. The suspension was filtered, and the filtrate was concentrated using a rotavapor, to give 130 g of crude extract. Part of this extract (100 g) was subjected to column chromatography over silica gel (Merck, 70 – 230 mesh) and eluted with a hexane-AcOEt mixture of increasing polarity. A total of 290 fractions of 300 mL each were collected and combined on the basis of their TLC profiles to afford eleven main fractions, F1–F11. Fractions F2: 80 – 95 (40 mg), F5: 140 – 155 (28 mg), F8: 181 – 230 (1.5 g) and F10: 255 – 267 (500 mg) were left to crystallize at room temperature to afford isoformononetin (**4**, 34 mg), 8-methoxyisoformonetin (**2**, 20 mg), amphiisoflavone (**1**, 800 mg) and 6-methoxyisoformononetin (**3**, 300 mg), respectively.

*Amphiisoflavone (**1**)*

Yellow needles, m. p.: 190 – 192 °C. – ^{1}H NMR see Table 2. – ^{13}C NMR see Table 1. – HRMS ((+)-ESI-TOF): $m/z = 627.1615$ (calcd. 627.1682 for $C_{35}H_{28}O_{10}K$, $[M+K]^+$). – EI-MS (70 eV): $m/z(\%) = 313$ (74), 298 (100), 297 (80), 283 (44), 166 (44), 132 (40).

*8-Methoxyisoformononetin (**2**)*

Colorless powder. – ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 8.40$ (s, 1 H, H-2), 7.70 (d, $J = 8.5$ Hz, 1 H, H-5), 7.00 (d, $J = 8.5$ Hz, 1 H, H-6), 7.50 (d, $J = 8.7$ Hz, 2 H, H-2′, H-6′), 6.90 (d, $J = 8.7$ Hz, 2 H, H-3′, H-5′), 3.80, 3.90 (2 s, 3 H each, 2 OMe). – ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta =$ 154.7 (C-2), 122.9 (C-3), 174.7 (C-4), 120.7 (C-5), 113.6 (C-6), 154.7 (C-7), 134.7 (C-8), 150.6 (C-9), 117.4 (C-10), 124.1 (C-1′), 130.0 (C-2′, C-6′), 115.2 (C-3′, C-5′), 158.9 (C-4′), 55.1, 60.7 (2 OMe). These ^{1}H and ^{13}C NMR data match well with the literature values [10]. – HRMS ((+)-ESI-TOF): $m/z = 299.0950$ (calcd. 299.0841 for $C_{17}H_{14}O_5$, $[M+H]^+$).

*6-Methoxyisoformononetin (**3**)*

Brown powder. – ^{1}H NMR see Table 2. – ^{13}C NMR see Table 1. ^{1}H and ^{13}C NMR data match well with the literature values [9]. – HRMS ((+)-ESI-TOF): $m/z = 299.0694$ (calcd. 299.0841 for $C_{17}H_{14}O_5$, $[M+H]^+$).

*Isoformononetin (**4**)*

Colorless needles, m. p.: 254 – 256 °C (lit.: 257 – 259 °C [13]). – IR (KBr) cm^{-1}: $\nu = 3151$ (–OH), 1644 (–C=O), 1611, 1532 (arom). – ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 8.32$ (s, 1 H, H-2), 7.85 (d, $J = 1.5$ Hz, 1 H, H-5), 6.92 (dd, $J = 8.5$ Hz, 1.5 Hz, 1 H, H-6), 6.85 (d, $J = 8.5$ Hz, 1 H, H-8), 7.50 (d, $J = 8.5$ Hz, 2 H, H-2′, H-6′), 6.98 (d, $J = 8.5$ Hz, 2 H, H-3′, H-5′), 3.81 (s, 3 H, OMe). – ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 153.2$ (C-2), 123.1 (C-3), 174.6 (C-4), 127.3 (C-5), 113.6 (C-6), 162.9 (C-7), 102.1 (C-8), 157.5 (C-9), 116.5 (C-10), 130.1 (C-2′, C-6′), 113.6 (C-3′, C-5′), 159.0 (C-4′), 55.2 (OMe). ^{1}H and ^{13}C NMR data match well with the literature values [11 – 13]. – HRMS ((+)-ESI-TOF): $m/z = 269.0600$ (calcd. 269.0735 for $C_{16}H_{12}O_4$, $[M+H]^+$).

*Acetylation of compound (**3**)*

20 mg of compound **3** was treated with AC_2O-pyridine (1 : 1) (3 mL). The mixture was stirred at room temperature for 12 h. The usual work-up and purification on a silica gel column afforded 10 mg of **3a** as colorless needles in 44 % yield.

*4′-Acetoxy-6,7-dimethylisoflavone (**3a**)*

M. p.: 164 – 165 °C. – ^{1}H NMR see Table 2. – ^{13}C NMR see Table 1.

*3-3-2 O-Prenylation of compound **3***

To a solution of **3** (20 mg) in acetone (10 mL) were added successively 20 mg of potassium carbonate and 0.3 mL of dimethylallylbromide. The reaction mixture was stirred at 70 °C for 3 h. After cooling, filtration and evaporation 12 mg of compound **3p** were obtained as colorless needles with 49 % yield.

*4′-O-Prenyl-6,7-dimethylisoflavone (**3p**)*

M. p.: 168 – 170 °C. – ^{1}H NMR see Table 2. – ^{13}C NMR see Table 1.

*Methylation of compound **3***

20 mg of compound **3** was dissolved in acetone (10 mL). To the resulting solution, 20 mg of potassium carbonate and 0.3 mL of dimethylsulfate were added successively. The reaction mixture was heated for 3 h at 70 °C under stirring. After cooling, filtration and concentration, compound **3m** (8 mg) was obtained as a colorless powder in 38 % yield.

*4′,6,7-Trimethoxyisoflavone (**3m**)*

M. p.: 180 – 182 °C (lit.: 180 – 182 °C [7]). – ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 8.30$ (s, 1 H, H-2), 7.40 (s, 1 H, H-5), 7.10 (s, 1 H, H-8), 7.47 (d, $J = 8.7$ Hz, 2 H, H-2′, H-6′), 6.90 (d, $J = 8.7$ Hz, 2 H, H-3′, H-5′), 3.78, 3.84, 3.90 (3 s, each 3 H, 3 OMe); the ^{1}H NMR data match well with the literature values [7, 9]. – ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 151.7$ (C-2), 124.1 (C-3), 174.7 (C-4), 103.7 (C-5), 148.2 (C-6), 153.4 (C-7), 100.0 (C-8), 152.0 (C-9), 117.9 (C-10), 124.0 (C-1′), 128.7 (C-2′, C-6′), 113.9 (C-3′, C-5′), 159.4 (C-4′), 55.8, 56.1, 56.3 (3 OMe); the ^{13}C NMR data match well with the literature values [12].

Bioassays

Antimicrobial assays

The studied microorganisms included reference (ATCC) and multidrug resistant strains of *Providencia stuartii* (NAE16), *Pseudomonas aeruginosa* (*PA01, PA124*), *Klebsiella pneumoniae* (*KP55*), *Escherichia coli* (*AG102*), *Enterobacter aerogenes* (*ATCC13048, EA27, CM64*), and *Candida albicans* ATCC 9002 obtained from the American Type Culture Collection. They were maintained on agar slant at 4 °C and sub-cultured on a fresh appropriate agar plate 24 h prior to the antimicrobial test. Nutrient agar and Sabouraud glucose agar were used for the activation of bacteria and fungi, respectively. The Mueller Hinton Broth (MHB) was used for MIC and MMC determinations. The Mueller Hinton Agar (MHA) was also used for the determination of the MMC [15].

Chloramphenicol (Sigma-Aldrich, St. Quentin Fallavier, France) was used as reference antibiotic (RA) against bacteria. *p*-Iodonitrotetrazolium chloride (I9NT, Sigma-Aldrich) was used as microbial growth indicator [16, 17].

The MIC determinations on bacteria were conducted using the rapid INT colorimetric assay according to described methods [16, 17] with some modifications. Briefly, the test sample was first dissolved in 10% (v/v) DMSO-MHB to give a final concentration of 512 μg mL^{-1} and serially diluted twofold to obtain concentration ranges. 100 μL of each concentration was added in a well (96-well microplate) containing 95 μL of MHB and 5 μL of inoculum (standardized at 1.5×10^6 CFU mL^{-1} by adjusting the optical density to 0.1 at 600 nm, Shimadzu UV-120-01 spectrophotometer) [18]. The final concentration of DMSO in the well was less than 3% (preliminary analyses with 3% (v/v) DMSO did not alter the growth of the test organisms). The negative control well consisted of 195 μL of MHB and 5 μL of the standard inoculum [19]. The plates were covered with a sterile plate sealer, then agitated to mix the contents of the wells using a plate shaker and incubated at 37 °C for 24 h. The assay was repeated trice. The MIC of samples was detected following addition (40 μL) of 0.2 mg mL^{-1} *p*-iodonitrotetrazolium chloride and incubation at 37 °C for 30 min [16, 17]. Viable microorganisms reduced the yellow dye to a pink color. MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of bacterial growth [20].

Antioxidant investigation: DPPH assay method

The free radical scavenging activity of the methanol extract and pure compounds was evaluated as described by Mensor *et al.* [21]. Briefly, the test samples, prior dissolved in DMSO (SIGMA), were mixed with a 0.3 mmol 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) ethanol solution, to give final concentrations of 5, 10, 50, 100, 500 and 1000 μg mL^{-1} (sample per microlitters of DPPH solution). After 30 min at room temperature, the absorbance values were measured at 517 nm and converted into percentage of antioxidant activity. Ascorbic acid was used as a standard control. Each assay was repeated trice, and the results recorded as mean of the triplicated experiments were graphically illustrated (Fig. 2). The inhibition ratio (%) was calculated as follows: % inhibition = [(absorbance of control − absorbance of test sample)/absorbance of control] × 100.

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