

Antimicrobial and radical scavenging flavonoids from the stem wood of *Erythrina latissima*

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Abstract

From the stem wood of *Erythrina latissima*, two isoflavones and a flavanone were isolated and characterized as 7,3'-dihydroxy-4'-methoxy-5'-(γ,γ -dimethylallyl)isoflavone (erylatissin A), 7,3'-dihydroxy-6'',6''-dimethyl-4'',5''-dehydropyrano [2'',3'': 4',5']isoflavone (erylatissin B), (–)-7,3'-dihydroxy-4'-methoxy-5'-(γ,γ -dimethylallyl)flavanone (erylatissin C), respectively, in addition to 10 known flavonoids. Structures of these compounds were determined on the basis of their spectroscopic data. These compounds showed antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Candida mycoderma*. The isolated compounds also exhibited weak radical scavenging properties towards DPPH radical.

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1. Introduction

The genus *Erythrina* comprises over 110 species showing cosmopolitan distribution (Joubert, 1998). Previous investigations of a number of species have shown them to be good source of C-prenylated flavones, isoflavones, isoflavanones and pterocarpanes (Barron and Ibrahim, 1996; Dewick, 1994) and of nitrogenous compounds (Bisby et al., 1994).

Erythrina latissima E. Meyer (Fabaceae-Papilionoideae) is a tree 9–24 m tall, found in Botswana, Zimbabwe and South Africa and whose root and stem are burnt and used for dressing open wounds (Van Wyk and Van Wyk, 1998). Previous studies on the seeds have yielded *Erythrina* alkaloids (Wanjala and Majinda, 2000a,b), while the pods, root bark and stem bark

yielded alkaloids, stilbenoids, lignans and flavonoids (Wanjala et al., 2002; Wanjala and Majinda, 2000a,b, 2001). In this paper, we present results of an investigation of the stem wood.

2. Results and discussion

The chloroform extract of the stem wood of *Erythrina latissima* was subjected to a succession of chromatographic procedures, including silica gel chromatography, gel permeation chromatography using Sephadex LH-20 and preparative TLC to afford three pure isolates 1, 2 and 3.

The EI-MS spectrum of compound 1 showed a molecular ion peak at m/z 352.1 corresponding to the molecular formula $C_{21}H_{20}O_5$. The ^{13}C NMR spectra gave 21 carbon signals and the DEPT and HMQC spectra confirmed that eleven of these were protonated carbons. The DEPT spectrum further showed one

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methoxy, two methyls, one methylene and seven methine carbons. A singlet proton observed at δ_{H} 8.14 (H-2) with a corresponding carbon resonance at δ_{C} 153.1 (C-2) in the ^1H and ^{13}C spectra respectively indicated that compound **1** is an isoflavone. The nature and identity of the isoflavone was evident from the ^1H NMR spectrum (Table 1) which showed the presence of a pair of *meta* coupled aromatic protons at δ_{H} 7.07 (*d*, $J = 2.1$ Hz) and δ_{H} 6.90, (*d*, $J = 2.1$ Hz) and an ABD aromatic spin system at δ_{H} 8.06, (*d*, $J = 8.7$ Hz), δ_{H} 7.00, (*dd*, $J = 8.7$, 2.1 Hz) and δ_{H} 6.91, (*d*, $J = 2.1$ Hz). The presence of a methoxy was evident from the sharp 3H signals at δ_{H} 3.82 ppm. The presence of a γ,γ -dimethylallyl (3-methylbut-2-enyl) group was evident from the two allylic methyl signals (δ_{H} 1.75, δ_{H} 1.72), one methylene doublet (δ_{H} 3.36, $J = 7.2$ Hz) and 1H allylic triplet (δ_{H} 5.32, $J = 7.2$). In addition, the EI-MS spectral fragmentation ions at m/z 137 (A_1) caused by *retro* Diels–Alder cleavage of the C-ring (Nkengfack et al., 1989) shows that ring A has only one hydroxyl group and no other substituents.

The HMBC data showed a proton at δ_{H} 8.06 to have a long-range correlation with C-4 (δ_{C} 175.0), C-7 (δ_{C} 162.7) and C-9 (δ_{C} 158.3). This observation confirmed that an ABD aromatic spin system is on A-ring and hence *meta* coupled aromatic protons are on B-ring. The ^{13}C NMR chemical shift values (δ_{C} 146.0 and δ_{C} 150.0) of the sp^2 carbon atoms in B-ring requires oxy-

genation to occur on adjacent carbons viz at C-3' and C-4'. Two *meta* coupled protons at δ_{H} 7.07 and δ_{H} 6.91 were assigned to H-2' (δ_{C} 115.7) and H-6' (δ_{C} 121.5) and hence the γ,γ -dimethylallyl group could only be placed at C-5' (δ_{C} 135.2). The H-6' proton (δ_{H} 6.91) showed an HMBC correlation with a carbon resonating at δ_{C} 150.0 (C-4') and the same carbon showed another correlation with methoxy protons resonating at δ_{H} 3.82, showing that the methoxy group at C-4' position. Further, the carbon chemical shift value of the methoxy group (δ_{C} 60.1) showed it to be in a hindered position (i.e., flanked by two *ortho di* substituents) thus confirming the position of the methoxy group to be at C-4' rather than C-3'. The rest of the structure was also confirmed through study of direct and long-range heteronuclear CH-coupling interactions. On the basis of these observations compound **1** was identified as 3',7-dihydroxy-4'-methoxy-5'-(γ,γ -dimethylallyl)isoflavone (5-deoxy-5'- γ,γ -dimethylallylpratenin) and named erylatissin A.

The EI-MS of compound **2** showed a molecular ion at m/z 336.2 consistent with the molecular formula $\text{C}_{20}\text{H}_{16}\text{O}_5$ with NMR signals characteristic an isoflavone nucleus. A comparison of its ^1H and ^{13}C (Table 1) NMR spectra with those of **1** revealed identical oxygenation at C-7, C-3' and C-4'. In compound **2**, however, signals due to 2,2-dimethylpyran (cyclised γ,γ -dimethylallyl unit) were observed instead of a normal γ,γ -dimethylallyl unit. The connectivity of the 2,2-dimethylpyran ring in ring B was deduced from HMBC data which showed a correlation between the vinylic protons at H-4'' with C-6', C-5' and C-4', and H-5'' showed the expected 3J correlation with C-5' implying that the 2, 2-dimethylpyran group is connected in ring B at C-4' and C-5' carbons. Compound **2** was therefore identified as 7,3'-dihydroxy-6'', 6''-dimethyl-4'',5''-dehydropyrano [2'',3'':4',5'] isoflavone (5-deoxysemilicoisoflavone B) and named erylatissin B.

The EI-MS of compound **3** showed a molecular ion peak at m/z 354.1 consistent with the molecular formula $\text{C}_{21}\text{H}_{22}\text{O}_5$. This was confirmed from ^{13}C NMR spectrum that showed twenty one carbon signals. A complete three proton spin system from the COSY spectrum, together with the three doublet of doublets in the ^1H NMR spectrum at δ_{H} 5.43 (H-2, *dd*, $J = 12.9$, 3.0 Hz), δ_{H} 3.00 (H-3a, *dd*, $J = 12.7$, 16.7 Hz) and δ_{H} 2.71 (H-3b, *dd*, $J = 16.7$, 3.0 Hz) indicates that ring C is saturated. Furthermore the α -O-substituted alkyl proton at δ_{H} 5.43 (H-2) indicates that compound **3** is a flavanone. Comparison of its ^1H and ^{13}C (Table 2) NMR with those of **1** revealed identical oxygenation pattern, with the methoxy group also at C-4'. The ^1H , ^{13}C , HMQC and HMBC spectra indicated the same substitution pattern as in compound **1** in both rings A and B. All natural (–)-flavanones have been shown to have S-chirality at C-2, using circular dichro-

Table 1

^1H (300 MHz) and ^{13}C (75.4 MHz) NMR chemical shifts for compounds **1** and **2** in acetone- d_6

Position	1		2	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
2	8.14, <i>s</i>	153.1	8.18, <i>s</i>	152.9
3		124.6		125.3
4		175.0		175.1
5	8.06, <i>d</i> (8.8)	127.9	8.08, <i>d</i> (8.8)	128.0
6	7.00, <i>dd</i> (8.7, 2.1)	115.4	7.01, <i>dd</i> (8.7, 2.2)	115.2
7		162.7		162.7
8	6.91, <i>d</i> (2.1)	102.7	6.92, <i>d</i> (2.2)	102.7
9		158.3		158.3
10		117.8		118.1
1'		128.7		121.8
2'	7.07, <i>d</i> (2.1)	115.7	7.06, <i>d</i> (2.0)	116.9
3'		150.0		145.5
4'		146.0		140.0
5'		135.2		124.6
6'	6.91, <i>d</i> (2.1)	121.5	6.87, <i>d</i> (2.0)	118.3
1''	3.36, <i>d</i> (7.2)	28.6		
2''	5.32, <i>t</i> (7.2)	123.7		76.9
3''		131.9	5.77, <i>d</i> (9.8)	131.4
4''	1.75, <i>s</i>	17.4	6.43, <i>d</i> (9.8)	122.6
5''	1.72, <i>s</i>	25.4	1.46, <i>s</i>	27.5
6''			1.46, <i>s</i>	27.5
4'-OCH ₃	3.82, <i>s</i>	60.1		

Assignments were confirmed by COSY, HMQC, HMBC and DEPT experiments.

ism spectroscopy (by observing the Cotton effect). The measured specific optical rotation for **3** was found to be -78° and thus from this value the absolute configuration at C-2 was assigned as 2S (Dewick, 1994). Compound **3** is thus identified (–)-7,3'-dihydroxy-4'-methoxy-5'-(γ,γ -dimethylallyl)flavanone (5-deoxysigmoidin B-4'-methyl ether) and named erylatissin C.

The genus *Erythrina* is known to elaborate various flavonoids classes and a recent review (Majinda et al., 2004) showed reported 76 isoflavones, 40 pterocarpanes, 34 flavanones, 27 isoflavanones, 6 isoflavans, 5 chalcones, 4 isoflav-3-enes and 3 flavonols. These metabolites, especially the isoflavones are fairly distributed in the genus. However, the isoflavones of the daidzein (5-deoxygenistein or 7,4'-dihydroxyisoflavone) derivatives to which erylatissins A **1** and B **2** belongs are relatively rare with only 10 having been so far reported. Six of these 10 are reported from *E. latissima*, with one or two each from *E. abyssinica* (**1**), *E. arborescens* (**1**), *E. eriotriochoa* (**1**), *E. indica* (**1**), *E. lysistemon* (**1**), *E. orientalis* (**1**), *E. poeppigiana* (**1**) *E. sigmoidea* (**2**), *E. suberosa* var *glabrescence* (**1**) *E. variegata* (**2**) and *E. x.bidwillii* (**1**). The flavanones of the liquiritigenin (5-deoxyflavanone or 7,4'-dihydroxyflavanone) class to which erylatissin C **3** belongs are rare with only five so far reported from the genus. Liquiritigenin, the simplest member, was isolated from *E. lysistemon* but the rest of the members, abyssinones I–IV, have been reported in *E. abyssinica* (all four), *E. latissima* (abyssinones II and IV), and *E. sigmoidea* [abyssinone IV] (Majinda et al., 2004). It thus appear that while daidzein derivatives are fairly distributed in the genus with the greatest number found in *E. latissima*, the liquiritigenin derivatives have so far been found in *E. abyssinica* and *E. latissima* and one report each in *E. lysistemon* and *E. sigmoidea*. The chemical types found in *E. abyssinica* and *E. latissima* seem to confirm the fact that the two are also morphologically very similar.

2.1. Antimicrobial and DPPH radical scavenging activity

The reported compounds were tested in vitro for their antimicrobial activities using rapid agar overlay or immersion bioautography (Rahalison et al., 1991; Rios et al., 1988). All the tested compounds were less active against Gram-negative bacteria than both Gram-positive bacteria and fungi (Table 3). The compounds tested were isoflavones (**1**, **2**, **4**, **6**, **7**, and **11**), flavanones (**3** and **5**), pterocarpanes (**8**, **9** and **13**) and chalcones (**10** and **12**). The most active compounds were **9**, **10** and **3** in that order. It seems that for chalcones the presence of a 3'-prenyl unit enhanced activity while for pterocarpanes the presence of a 6a-hydroxy and/or a 9-methoxy groups were important for activity and for flavanones 3',4'-hydroxylation and 5'-prenyla-

tion were essential for activity. The isolated compounds showed weak radical scavenging properties with compounds **8**, **10** and **4** being the most active. It seems for the pterocarpanes both the presence of 9-hydroxy and a 10-prenyl group were important for radical scavenging activity while it appears the presence of a 9-methoxy and/or a 6a-hydroxy reduced activity of the same, while for chalcones it appears the presence of a 3'-prenyl and 4'-hydroxy enhanced activity. Furthermore, it appears that for isoflavones the presence a 4'-hydroxylation and less bulky substituents in B-ring enhanced activity and that hydroxylation at either 7 only or both 5,7 did not seem to affect activity.

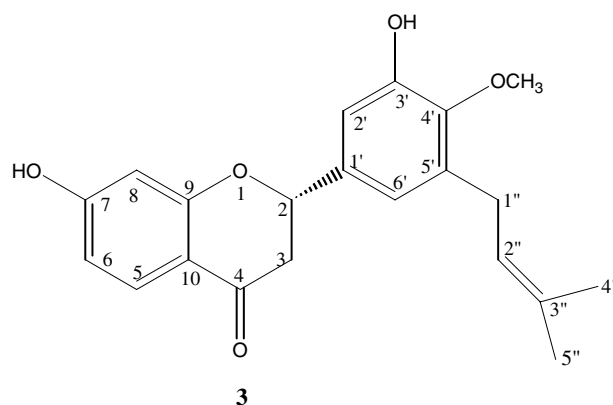
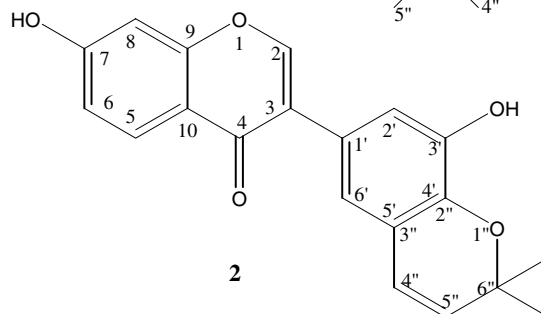
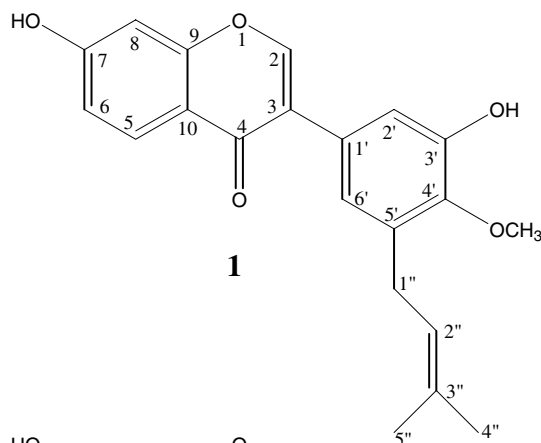


Table 2
¹H (300 MHz) and ¹³C (150 MHz) NMR chemical shifts for compound **3** in acetone-d₆

Position	δ _H	δ _C
2	5.43, 1H, <i>dd</i> (12.7, 3.0)	79.9
3a	3.00, 1H, <i>dd</i> (16.7, 12.7)	44.2
3b	2.71, 1H, <i>dd</i> (16.7, 3.0)	44.2
4		190.0
5	7.73, 1H, <i>d</i> (8.6)	128.9
6	6.59, 1H, <i>dd</i> (8.6, 2.2)	110.8
7		165.3
8	6.45, 1H, <i>d</i> (2.2)	103.2
9		163.9
10		114.3
1'		135.6
2'	6.97, 1H, <i>d</i> (2.0)	112.9
3'		150.0
4'		146.2
5'		135.7
6'	6.87, 1H, <i>d</i> (2.0)	119.1
1''	3.38, 2H, <i>d</i> (7.2)	28.7
2''	5.31, 1H, <i>t</i> (7.2)	123.4
3''		131.8
4''	1.75, 3H, <i>s</i>	17.4
5''	1.73, 3H, <i>s</i>	25.4
4'-OCH ₃	3.82, 3H, <i>s</i>	60.2

Assignments were confirmed by COSY, HMQC, HMBC and DEPT experiments.

3. Experimental

3.1. General

M.ps uncorrected. UV: Shimadzu UV-2501PC spectrophotometer. IR: Perkin–Elmer 2000 FT-IR spectrometer. ¹H NMR, ¹³C NMR, DEPT, COSY, HMQC, HMBC: Bruker Avance DPX 300 and DRX

600 spectrometers using standard pulse sequences and referenced to residual solvent signals. EI-MS: Finnigan MAT SSQ 7000 Single Quadrupole Instrument at 70 eV. CC: silica gel 60 (0.040–0.063 mm, Merck). Preparative and analytical TLC: silica gel 60 PF₂₅₄₊₃₆₆ (Merck). Visualisation of chromatograms: UV (254 and 366 nm) and Vanillin–sulphuric acid spray.

3.2. Plant material

Stem wood of *E. latissima* was collected in Mapoka Village, North East District, Botswana. The species was identified by Dr. L.M. Turton and a voucher specimen (E 0897) is deposited at the University of Botswana Herbarium.

3.3. Extraction and isolation

The dry and powdered stem wood (1.4 kg) was extracted with CHCl₃/MeOH (1:1) mixture and removal of solvent from the extract gave a brown residue (352.0 g). The extract was dissolved in water and partitioned successively between CHCl₃ and *n*-BuOH. The CHCl₃ extract (266.2 g) was adsorbed on 150.0 g of silica gel and applied to a normal column packed with 250.0 g of silica gel using CHCl₃. The column was eluted using the following solvent systems: CHCl₃/EtOAc (6:1)-fractions 1–5, CHCl₃/EtOAc (6:2)-fractions 6–8, CHCl₃/EtOAc (6:3)-fractions 9–12, EtOAc-fractions 13–19.

Fractions that showed similar components on TLC were combined together as follows: 4–11 and 14–19. The combined fraction 14–19 (30.0 g) was adsorbed in

Table 3
 The antibacterial, antifungal and radical scavenging activities of the compounds isolated from the root wood of *Erythrina latissima*

Compound	Test organisms (minimum inhibitory amount of compound in µg)				DPPH assays	
	<i>E. coli</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>C. mycoderma</i>	TLC (µg)	IC ₅₀ (µg/ml)
1	5.00	0.10	0.10	0.02	0.5	780
2	NA	1.00	1.00	1.00	10	>1000
3	0.50	0.10	0.01	0.01	0.5	710
4	NA	1.00	5.00	0.05	0.1	380
5	10.00	0.50	0.50	0.01	0.5	630
6	NA	50.00	50.00	0.05	NT	NT
7	0.50	0.10	0.10	0.02	0.5	671
8	5.00	0.50	0.50	0.10	0.1	135
9	0.10	0.01	0.01	0.05	1.0	>1000
10	0.50	0.01	0.01	0.05	0.1	160
11	100.00	1.00	5.00	0.10	0.5	354
12	1.00	0.10	0.10	0.50	0.5	810
13	10.00	1.00	1.00	0.50	0.5	450
Reference	Chl	Chl	Chl	Miconazole	Qu < 0.05	Qu 7
Compounds	0.001	0.0001	0.0001	0.0001	Ga < 0.05	Ga 4
					Aa < 0.10	Aa 18

NA, no activity up to 100.00 µg; NT, not tested; Chl, chloramphenicol; Qu, quercetin; Ga, gallic acid; Aa, ascorbic acid. Results are recorded as means of three readings.

30.0 g of silica gel and applied on a column packed with 80.0 g of silica gel using $\text{CHCl}_3/\text{EtOAc}$ (6:2) and run with the same solvent system to obtain 7,4'-dihydroxyisoflavone (daidzein), **4** (15.9 mg) (Dewick, 1994; Yu et al., 2000).

The combined fraction 4–11 (108.2 g) was adsorbed in 100 g of silica gel and applied on a normal column packed with 200 g of silica gel using CHCl_3 . Thirty nine fractions were collected and the solvent systems used to elute the column were CHCl_3 (100%)—fractions 1–18, $\text{CHCl}_3/\text{EtOAc}$ (9:1)—fractions 19–27, $\text{CHCl}_3/\text{EtOAc}$ (6:2)—fractions 28–36 and EtOAc (100 %)—fractions 37–39. These fractions, based on TLC, were combined as follows: fraction A (fractions 1–13), B (fractions 14–19), C (fractions 20–32) and D (fractions 33–39).

The combined fraction B was applied on the preparative TLC and eluted with toluene/ EtOAc/AcOH (45:4:1) ($\times 3$ developments) to give 7,4'-dihydroxy-3'- γ,γ -dimethylallylflavanone (abyssinone II) **5** (123.6 mg) (Kamat et al., 1981).

The combined fraction C was passed through Sephadex LH-20 ($\text{CHCl}_3/\text{MeOH}$, 1:1) to give fractions C-1, C-2 and C-3. Fraction C-1 was applied on preparative TLC eluted with toluene/ EtOAc/AcOH (40:9:1) ($\times 4$ developments) to give 7,3'-dihydroxy-4'-methoxyisoflavone (calycosin) **6** (34.4 mg); 7,4'-dihydroxy-3'- γ,γ -dimethylallyl isoflavone (neobavaisoflavone) **7** (77.4 mg) (Nkengfack et al., 1994); 7,3'-dihydroxy-4'-methoxy-5'-prenylflavanone **3** (80.0 mg) and 7,3'-dihydroxy-4'-methoxy-5'- γ,γ -dimethylallyl isoflavone **1** (24.0 mg).

Fraction C-2 was applied on preparative TLC eluted with toluene/ EtOAc/AcOH (40:9:1) [$\times 3$] to give band II and I. Both bands were re-applied on preparative TLC eluted with toluene/ EtOAc/AcOH (45:4:1) [$\times 5$] to give compounds 7,3'-dihydro-6'', 6''-dimethyl-4'', 5''-dehydropyrano[2'',3'':4',5']isoflavone **2** (5.0 mg) and 3,9-dihydroxy-10- γ,γ -dimethylallylpterocarpan (phaseollidin) **8** (4.7 mg) (Mitscher et al., 1988) from band I and 3,6-dihydroxy-9-methoxy-10- γ,γ -dimethylallylpterocarpan (crisatcarpin) **9** (103.9 mg) (Fomum et al., 1986; Tanaka et al., 1996) from band II.

Fraction C-3 was passed through Sephadex LH-20 (EtOAc) to give fraction C-3-1 and C-3-2. Fraction C-3-1 was applied on the preparative TLC eluted with toluene/ EtOAc/AcOH to give 4,2',4'-trihydroxy-3'- γ,γ -dimethylallylchalcone **10** (12.2 mg) (Asada et al., 1998). Fraction C-3-2 (20.0 g) was adsorbed in 20.0 g of silica gel and applied to a column packed with 50.0 g of silica gel using toluene/ EtOAc/AcOH (40:9:1) and eluting with the same solvent system to give 5,7,4'-trihydroxyisoflavone (genistein) **11** (132.2 mg) (Asres et al., 1985) and fraction C-3-2-1. Fraction C-3-2-1 was applied on preparative TLC eluted with toluene/ EtOAc/AcOH (45:4:1) to give 4,2',4'-trihydroxychalcone **12** (50.1 mg) (Bohm, 1994) and 3,9-dihydroxy-

pterocarpan (demethylmedicarpin) **13** (4.7 mg) (Dewick, 1994; Huang and Liou, 1997).

3.3.1. 7,3'-Dihydroxy-4'-methoxy-5'- γ,γ -dimethylallylisoflavone (erylatissin A), **1**

Brown paste. UV: λ_{max} MeOH nm ($\log \epsilon$): 310 (sh), 249 (4.2); +NaOMe 334 (sh), 260; + AlCl_3 309 (sh), 247; + AlCl_3/HCl 310, 249; +NaOAc 336 (sh), 265; +NaOAc/ H_3BO_3 248. IR (KBr pellets): ν_{max} cm^{-1} 3414, 2923, 1624, 1509, 1290. EI-MS: m/z (rel. int.), 352 (60) $[\text{M}]^+$, 337 (38), 137 (100) (MF $\text{C}_{21}\text{H}_{20}\text{O}_5$).

3.3.2. 7,5'-Dihydroxy-6'',6''-dimethyl-4'',5''-dehydropyrano[2'',3'':4',5']isoflavone (erylatissin B), **2**

Yellowish paste. UV: λ_{max} MeOH nm ($\log \epsilon$): 312 (sh), 256 (4.10); +NaOMe 335 (sh), 275; + AlCl_3 314 (sh), 256; + AlCl_3/HCl 313 (sh), 257; +NaOAc 334 (sh), 276; +NaOAc/ H_3BO_3 256. IR (KBr pellets): ν_{max} cm^{-1} 3415, 2925, 1618, 1250. EI-MS: m/z (rel. int.) 336 (100) $[\text{M}]^+$, 199 (16), 137 (51) (MF $\text{C}_{20}\text{H}_{16}\text{O}_5$).

3.3.3. (–)-7,3'-Dihydroxy-4'-methoxy-5'- γ,γ -dimethylallylflavanone (erylatissin C), **3**

Yellow solid, m.p. 56–59 °C, $[\alpha]_{\text{D}} -78^\circ$ (MeOH, c 0.025). UV: λ_{max} MeOH nm ($\log \epsilon$): 329 (sh), 279 (4.10); +NaOMe 337; + AlCl_3 328 (sh), 279 (4.10); + AlCl_3/HCl 280; +NaOAc 335, +NaOAc/ H_3BO_3 329. IR (KBr pellets): ν_{max} cm^{-1} 3414, 2924, 1618, 1249. EI-MS: m/z (rel. int.) 354 (93) $[\text{M}]^+$, 269 (100), 217 (15), 137 (46) (MF $\text{C}_{21}\text{H}_{22}\text{O}_5$).

3.4. Antibacterial and antifungal assays

The activity tests were done using the TLC bioautographic technique using the standard procedures by Rahalison et al., 1991; against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Candida mycoderma*. The standards used for comparison were chloramphenicol for bacteria and miconazole for fungus. Exact procedure is as previously reported (Bojase et al., 2002; Wanjala et al., 2002). The experiments were run in triplicate.

3.5. DPPH assays

Reduction of DPPH (2,2-diphenyl-1-picrylhydrazyl or 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazyl) radical [molecular formula $\text{C}_{18}\text{H}_{12}\text{N}_5\text{O}_6$, Mwt 394]. TLC autographic assay: after developing and drying, TLC plates (with amounts of sample ranging from 0.1 to 100 μg) were sprayed with 0.2% (2 mg/ml) of DPPH solution in methanol. The plates were examined half an hour after spraying. Active compounds appeared as yellow spots against a purple background (Cuendet et al., 1997, 2000; Takao et al., 1994). The samples were

done in triplicate and the mean value of the three was recorded.

One millilitre of 500 μ M (0.2 mg/ml) DPPH in methanol was mixed with equal volumes of test compounds at various concentrations, mixed well and kept in the dark for 30 min. The absorbance at 517 nm was monitored in the presence of different concentrations of the samples. Blank experiment was also carried out, with just solvent and DPPH (i.e., 2 ml of 500 μ M in methanol), to determine the absorbance of DPPH before interacting with the compounds. The amount of sample in μ g/ml at which the absorbance at 517 nm decreases to half its initial value was used as the IC₅₀ value of the compound (Naik et al., 2003). The samples were done in triplicate and the mean value of the three was recorded.

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