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Cytotoxic benzil and coumestan derivatives from *Tephrosia calophylla*

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ABSTRACT

A benzil, calophione A, 1-(6′-Hydroxy-1′,3′-benzodioxol-5′-yl)-2-(6″-hydroxy-2″-isopropenyl-2″,3″-dihydro-benzofuran-5″-yl)-ethane-1,2-dione and three coumestan derivatives, tephcalostan B, C and D were isolated from the roots of *Tephrosia calophylla*. Their structures were deduced from spectroscopic data, including 2D NMR ^{1}H–^{1}H COSY and ^{13}C–^{1}H COSY experiments. Compounds were evaluated for cytotoxicity against RAW (mouse macrophage cells) and HT-29 (colon cancer cells) cancer cell lines and antiprotozoal activity against various parasitic protozoa. Calophione A exhibited significant cytotoxicity with IC_{50} of 5.00 (RAW) and 2.90 μM (HT-29), respectively.

1. Introduction

In continuation of our chemical investigation on some unexplored Indian *Tephrosia* species (Ganapaty et al., 2003, 2008), with our focus on the search for new antitumour and antiprotozoal leads, we have examined *Tephrosia calophylla*. BEDD (Fabaceae), a perennial under-shrub found widely distributed in the forests of Talakona, Andhra Pradesh, South India (Thammanna et al., 1994). Earlier investigations on *T. calophylla* have revealed the isolation of a coumestan derivative and two known flavonoids (Kishore et al., 2003). We report herein the isolation, characterization, *in vitro* cytotoxicity against RAW (mouse macrophage cells) and HT-29 (colon cancer cells) cancer cell lines and antiprotozoal activity (Table 3) of one novel benzil and three new coumestan derivatives from the roots of *T. calophylla*. Their structures were deduced from spectroscopic data, including 2D NMR, ^{1}H–^{1}H COSY and ^{13}C–^{1}H COSY experiments (Tables 1 and 2).

2. Results and discussion

Extensive silica gel column chromatography of the chloroform extract of the roots of *T. calophylla* using solvents of increasing polarity afforded *n*-hexane, chloroform ($CHCl_3$) and methanol (MeOH) soluble fractions. The separation of the chloroform soluble fractions with silica gel open-column chromatography, and repeated crystallization led to the isolation of a benzil and three coumestan derivatives (**1**–**4**).

The molecular formula of $C_{20}H_{17}O_7$ (*m*/*z*) 369.09698 $[M+H]^+$) was established for calophione A (**1**) by the HRESIMS data. The ^{13}C NMR displayed resonances for two carbonyl carbons (δ_C 194.0 and 193.5) consistent with a benzil derivative reported earlier from *Derris scandens* (Mahabusarakam et al., 2004), together with signals corresponding to an oxymethine carbon (δ_C 88.3), an upfield methylene (δ_C 32.6), eight quartenary carbons δ_C 168.5, 167.6, 164.3, 156.4, 141.2, 120.3, 110.6, 109.4 and a down-field sp^3 carbon δ_C 102.4, confirming **1** to be associated with a benzil moiety, a benzofuran and a benzodioxy functionality (Mahabusarakam et al., 2004; Chien-Chang et al., 2006). This observation was further supported by ^{1}H NMR spectra that displayed resonances for two hydrogen-bonded hydroxyls at δ_H 12.24 (OH-6′) and δ_H 12.06 (OH-6″), a singlet resonance for a methylenedioxy group at δ_H 6.00 (H-2′) and four well separated one-proton singlets for aromatic protons, δ_H 7.23 (H-4″), δ_H 6.82 (H-4′), δ_H 6.53 (H-7′) and δ_H 6.45 (H-7″) clearly indicative that **1** consisted of two tetra-substituted rings (Ferrari et al., 1984). In addition, the NMR spectrum exhibited an ABX system for a 2″,3″a,7″a-tri-substituted dihydrofuran ring comprising of an oxymethine at δ_H 5.30 *dd* (*J* = 10.6, 8.7) coupled to the *gem*-methylene protons at δ_H 3.30 *dd* (*J* = 15.6, 10.6), 2.90 *dd* (*J* = 15.6, 8.7) and signals for an exomethylene, δ_H 4.98 and 5.10 as singlets and a methyl singlet at δ_H 1.72 constituting the presence of an isopropenyl unit as part of the side chain. This was further confirmed from the spin systems observed

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Table 1
^{1}H NMR, ^{13}C NMR and COSY data of calophione A **1** (δ values in $CDCl_3$).

C/H	δ Values		Mult (*J*, Hz)	COSY
	^{13}C	^{1}H		
1-CO	194.0			
2-CO	193.5			
2′	102.4	6.00	*S*	
3′a	141.2			
4′	108.0	6.82	*S*	
5′	109.4			
6′	167.6[b]			
6′–OH		12.24		
7′	98.9[a]	6.53	*S*	
7′a	156.4			
2″	88.3	5.30	*dd* (10.6, 8.7)	H-3″ β
3″α/β	32.6	3.30	*dd* (15.6, 10.6)	H-3″ β
		2.90	*dd* (15.6, 8.7)	
3″a	120.3			
4″	128.0	7.23	*br s*	
5″	110.6			
6″	168.5[b]			
6”–OH		12.06	*S*	
7”	98.5[a]	6.45	*S*	
7”a	164.3			
1”′	142.7			
2”′a/b	113.2	5.10	*br s*	
3”′–CH_3	16.9	1.72	*S*	

[a,b] Assignments may be interchangeable due to proximity of signals.

in the COSY experiment of **1** (see Table 1). Further evidence regarding the connectivities of the subunits A and B was accomplished using the HMBC experiment (Fig. 1). For instance, the H-4′ methine (subunit-A) exhibited ^2J, ^3J correlations with the quaternary carbon at C-5′ (δ_C 109.4), the carbonyl (δ_C 194.0) and a second quaternary carbon at C-3′a (δ_C 141.2), while the H-7′ methine showed ^2J coupling with C-6′ (δ_C 167.6) suggesting that the latter was oxygenated. The placement of the methylenedioxy group at C-7′a/3′a of the subunit B was confirmed from HMBC correlations between the methylene protons (H_2-2′) and the quaternary carbons C-3′a and C-7′a. Similarly, HMBC correlations were observed (subunit-B) between the H-4″ methine (δ_H 7.23) and a second carbonyl (δ_C 193.5), the quaternary carbon at C-3″a (δ_C 120.3) and the H-7″ methine and the quaternary carbons at C-6″ and C-7″a, indicating that H-4″/7″ remained unsubstituted. The location of the isopropenyl side chain at C-2″ of the dihydrobenzofuran ring was provided from correlations observed between the methyl protons (δ_H 1.72) and the oxymethine carbon at C-2″ (δ_C 88.3) and the sp^2 carbon (C-2‴, δ_C 113.2). On the basis of detailed analysis of the 2D NMR (COSY, HSQC, and HMBC) experiments, with the inter proton couplings established through the homonuclear COSY experiment, all the signals in the ^{1}H NMR and ^{13}C NMR spectra were carefully assigned (Table 1). Consequently, **1** was identified as a new benzil derivative, 1-(6′-hydroxy-1′,3′-benzodioxol-5′-yl)-2-(6″-hydroxy-2″-isopropenyl-2″,3″-dihydro-benzofuran-5″-yl)-ethane-1,2-dione, for which we propose the name calophione A (**1**).

The HR-ESIMS of compound **2** suggested a molecular formula $C_{20}H_{13}O_6$ (*m*/*z* = 349.07073 $[M+H]^+$). The ^{1}H and ^{13}C NMR data (Table 2) closely resembled those of tephcalostan (Kishore et al., 2003), a coumestan derivative isolated previously from *T. calophylla*. However, the presence of a deshielded signal for an olefinic double bond at δ_H 6.08 located in the side chain suggested the presence of an ethenyl side unit. Further confirmation was obtained from HMBC correlations between the exocyclic methylene protons at δ_H 5.28/5.45 (H-7′a,b) and the olefinic carbon C-6′ (δ_C 136.5) and a ^2J coupling between the oxymethine, δ_H 5.43 (H-5′) of the dihydrofuran ring and the sp^2 carbon, δ_C 136.5. Thus, compound **2** was determined to be a new coumestan derivative, tephcalostan B.

Table 2
NMR data of coumestans (**2**–**4**) in *DMSO-d*$_6$ (^{1}H, 300 MHz; ^{13}C, 75 MHz).

C/H	2		3		4	
	δ_H (mult; *J* in Hz)	δ_C	δ_H (mult; *J* in Hz)	δ_C	δ_H (mult; *J* in Hz)	δ_C
1	7.80 (*s*)	116.7[a]				
2		125.0		160.6		160.6
3		162.1		102.3		103.6
3a				157.3		157.2
4	6.96 (s)	97.6		115.6[a]		159.8
5			7.37 (*s*)	94.1	7.44 (*s*)	94.3
4a		153.8				
6 CO		156.8				
6				145.5		145.7[a]
6a/b		116.0				
105.0						
7	7.28 (*s*)	94.0		146.5		146.8[a]
8		145.5	6.77 (*s*)	98.2	6.82 (*s*)	98.3[b]
8a				149.4		149.6[c]
9		146.7	6.10 (*s*)	101.9[b]	6.11 (*s*)	101.9
10	7.43 (*s*)	98.3				
10a		149.6				
11a		159.4				
11b		101.5				
1’				102.4[b]		115.7
2′				139.4		137.7
3′				148.0		148.2
4′	3.58 (*dd*, 15.1, 9.2) 3.13 (*dd*, 15.1, 7.3)	33.6		115.7[a]		94.3
5′	5.43 (*m*)	84.5		150.4		149.7[c]
6′	6.08 (*m*)	136.5	7.20 (*s*)	101.4	7.21 (s)	98.8[b]
7′	(a) 5.45 (*m*)	116.5[a]				
	(b) 5.28 (*m*)					
O–	CH_2–O	6.13 (*s*)	101.6			
2″				73.9		75.7
3″/4″			1.36 (*s*)	26.1	1.46 (*s*)	26.8
5″			1.90 (*t*, 6.8)	31.5	6.02 (*d*, 10.1)	133.3
6″			3.25 (*t*, 6.8)	20.9	7.32 (*d*, 10.1)	118.9

[a,b,c] Signals within a column are interchangeable and are represented correctly.

Compound **3** was assigned the molecular formula $C_{21}H_{17}O_7$ by HRESI-MS (*m*/*z* = 381.09671 $[M+H]^+$). 1D and 2D NMR spectra of **3** displayed characteristic signals for a coumestan derivative (Dewick, 1993; Kishore et al., 2003; Wang et al., 2006). The ^{1}H NMR spectrum in DMSO (Table 2) exhibited a singlet for 6H, assigned to a pair of magnetically equivalent methyl protons at δ_H 1.36, together with a triplet at δ_H 1.90 for a methylene and a second triplet at δ_H 3.25 for a deshielded methylene constituting the presence of a dimethylpyran residue in **3**. In addition to these, 1H singlets were observed for aromatic methines at δ_H 7.37, 6.77 and 7.20 assignable to H-5/8 (B-ring) and H-6′ (A-ring) of a coumestan skeleton (Kishore et al., 2003); a signal at δ_H 6.10 was due to a methylenedioxy group. The ^{13}C NMR spectrum (Table 2) exhibited 21 carbon resonances, i.e. a δ-lactone carbonyl at δ_C 160.6, three sp^3 carbons δ_C (20.9, 31.5, 101.9) two equivalent methyl carbons (δ_C 26.1), three sp^2 carbons (δ_C 94.1, 98.2, 101.4) and twelve quaternary carbons, while the HMBC experiment was used to establish ^2J and ^3J heteronuclear interactions (Fig. 2). The latter established the presence of a benzofuran moiety in **3**. Support for the structure of **3** was obtained from HMBC (^2J and ^3J) correlations between the H-5 methine and the quaternary carbons, C-4/6 (A-ring) and H-8 methine and C-7 (δ_C 146.5), while the methylenedioxy protons exhibited ^3J couplings to carbons C-6/7 establishing the placement of the methylenedioxy group at C-6/7 junction of the B-ring. Based

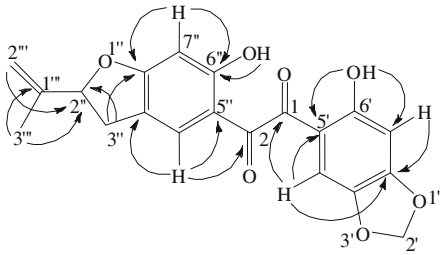
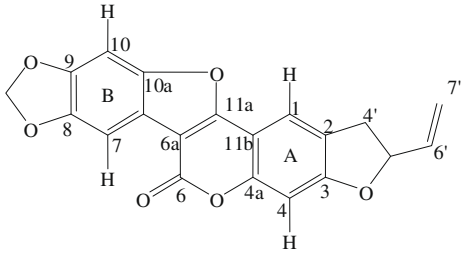
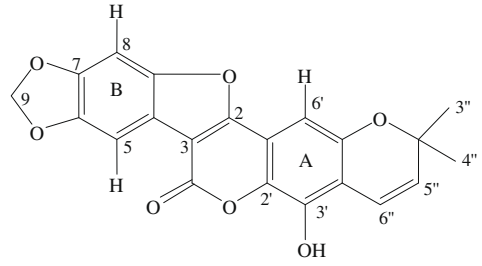


Fig. 1. Correlations observed with heteronuclear long-range coupling ^{13}C–^{1}H COSY spectra (HMBC) of **1**.



2



4

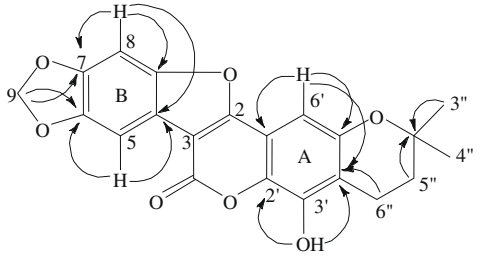


Fig. 2. Key HMBC correlations for **3**.

on the HMBC correlations (Fig. 2) between the hydroxyl proton and the quaternary carbons at C-3′/4′ (δ_C 148.0 and 115.7), the hydroxy group could be reasonably placed at C-3′ of the A-ring. Finally, the placement of the 2,2-dimethylpyran ring at C-4′/5′ (angular) with an oxygenated substituent at C-3′ (A-ring) was once again achieved through the HMBC experiment (H-6′–C-5′ (δ_C 150.4); H-6″ to C-4′; δ_C 115.7). These data unambiguously permitted the assignment of structure **3** for the new coumestan derivative, tephcalostan C.

Compound **4** afforded a *pseudo*molecular ion $[M+H]^+$ at m/z = 379 compatible with the molecular formula $C_{21}H_{15}O_7$. This was supported by NMR data, which were nearly comparable to that of **3** but displayed additional signals of an AX system at δ_H 6.02/7.32 (*d*, *J* = 10.1 Hz), corresponding to H-5″/H-6″ of the dimethyl chromene ring (Table 2). This allowed us to identify compound **4** as a new coumestan derivative, tephcalostan D. The ^{1}H and ^{13}C NMR data obtained for **3** and **4** are recorded in Table 2 and are reproduced here for the purpose of comparison with the other new coumestan derivative **2**.

Table 3
In vitro cytotoxicity (tumor cells) and antiprotozoal activity (IC_{50} values in μM) of compounds **1**–**4**[a].

Compounds	IC_{50} values (μM)						
	Cytotoxicity-tumor cells		Antiprotozoal activity				
	RAW	HT-29	*T. b. rhod*	*T. cruzi*	*L. don. axenic*	*P. falc. K1*	L-6 cells
1	5.00	2.90	–	–	–	–	–
2	10.00	10.00	–	–	–	–	–
3	9.70	15.40	16.68	>30	>90	>5	>90
4	17.00	6.47	17.83	>30	15.63	>5	>90
Standards	–	4.36	0.005	1.69	0.47	0.19	0.048

Standards used: amrubicin hydrochloride (HT-29 colon tumor cell line), melarsoprol (*T.b. rhodesiense*), benznidazole (*T. cruzi*), miltefosine (*L.donovani*), chloroquine (*P. falciparum*) and podophyllotoxin (L-6 cells, cytotoxicity).

[a] The inhibitory concentration of a compound/standard in μM necessary to achieve 50% growth inhibition (IC_{50}). Results obtained are values from two replicate experiments.

The *in vitro* cytotoxicity of the benzil and coumestan derivatives (**1**–**4**) was evaluated against RAW (mouse macrophage cells) and HT-29 (colon cancer cells) cancer cell lines using the MTT assay method (Mosmann, 1983 and Wilson, 2000). The results are presented in Table 3. Among the tested compounds, calophione A (**1**) exhibited significant cytotoxicity with IC_{50} of 5.00 (RAW), and 2.90 μM (HT-29) that was two-fold more potent in comparison to the known standard amrubicin hydrochloride (HT-29), while all three coumestan derivatives (**2**–**4**) exhibited moderate cytotoxicity against both RAW and HT-29 cell lines. Related phenolic compounds, isolated from different plants have been reported to exhibit significant antiprotozoal activities (Sauvain et al., 1994; Ayafor et al., 1997; Valsaraj 1997; Torres-Santos et al., 1999). Compounds **1** and **2** were taken up for several biological screening programs. Due to scarcity of the samples, we evaluated **3** and **4** for their *in vitro* antiprotozoal activity (Table 3) against parasitic protozoa, *Trypanosoma brucei rhodesiense* (STIB 900 strain, trypomastigote stage), *Trypanosoma cruzi* (Tulahuen strain C2C4, trypomastigote stage), *Leishmania donovani* (strain MHOM-ET/67/L82, amasti-gote stage) and *Plasmodium falciparum* (K1 strain, IEF stage, chloroquine resistant) using conventional procedures (Baltz et al., 1985; Orhan et al., 2006; Weniger et al., 2006; Brun and Schonenberger, 1979; Matile and Pink, 1990). Both **3** and **4** were found to be weakly active in comparison with standard drugs like melarsoprol (*T. b. rhodesiense*), benzindazole (*T. cruzi*), miltefosine (*L. donovani*), chloroquine (*P. falciparum*) and podophyllotoxin (L-6 cells, cytotoxicity).

3. Conclusions

In conclusion, one new benzil derivative, calophione A (**1**) and three new coumestan derivatives were isolated from the roots of *T. calophylla*. Benzil derivatives are rarely encountered in nature; nevertheless a small group of naturally occurring benzil derivatives was previously reported from *Iris tenuifolia, Zollernia paraensis, Glycyrrhiza glabra, Lespedeza homoloba* and *D. scandens* (Choudhary et al., 2008; Ferrari et al., 1984; Miyase et al., 1999; Li et al., 1998; Mahabusarakam et al., 2004) which is presumed to arise *via* oxidation of flavonoid precursors. Tephcalostan B (**2**), C (**3**) and D (**4**) are unique metabolites possessing a methylenedioxy group (subunit-B) and a prenyl side chain that readily cyclizes in certain cases to form a dimethylpyran/dihydrofuran ring (sub-unit-A), while oxygenation is restricted to C-3 of subunit-A. From the results of

the *in vitro* biological tests, the benzil and coumestan derivatives may be regarded as the cytotoxic principles of *T. calophylla*. Calophione A is worthy of consideration as a potential candidate in tumor and related diseases through further biological evaluation.

4. Experimental

4.1. General experimental procedures

Melting points were recorded on a Cipla I-28, digital apparatus and were uncorrected. Silica gel (Merck) 60–120 mesh for column chromatography and pre-coated TLC sheets (layer thickness 0.2 mm) and preparative TLC plate (layer thickness 1.25 mm) of silica gel 60 GF_{254} were used. Spots on chromatogram were detected under UV light and by spraying with 5% H_2SO_4 in methanol. ^1H (300 MHz) and ^{13}C NMR (125 MHz) spectra were measured on Varian Unity 300 and Varian Inova 500 spectrometers in $CDCl_3$ or DMSO, respectively, while the 2D NMR homo and heteronuclear experiments (COSY, HSQC and HMBC) were carried out on a Varian Inova 500 spectrometer at 499.8 and 125.7 MHz, respectively, in $CDCl_3$ or DMSO. High-resolution mass spectra (HRMS) were recorded by ESI MS on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA) with HP-Mix as standard.

4.2. Plant material

The root parts of *T. calophylla* were collected from Khailasa hills, Visakhapatnam, India, during February 2006. The sample was authenticated by Dr. M. Venkayya, Taxonomist, Department of Botany, College of Science and Technology, Andhra University. A voucher specimen (SG/TCR/06/113) has been deposited at the Herbarium, College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, India.

4.3. Extraction and isolation

Air-dried and finely powdered roots (1.5 kg) of *T. calophylla* were macerated at room temperature for 72 h with chloroform (3 × 4.5 l). Removal of the solvent under reduced pressure yielded 18.0 g of a crude extract, which was subjected to column chromatography on silica gel (60–120 mesh). Elution was started with pure hexane followed by a stepwise gradient of hexane–$CHCl_3$, and then $CHCl_3$–MeOH. A total of 24 fractions ($L1F_1$–$L1F_{24}$) were collected. Based on their TLC profiles, related fractions were pooled and then subjected again to silica gel chromatography eluting with hexane, hexane–$CHCl_3$ and $CHCl_3$–MeOH mixtures, to afford 12 fractions of each ca. 100 ml from $L2F_1$–$L2F_{12}$ (A–L). On crystallization using chloroform, fraction B afforded **1** (25 mg), mp 120–122 °C, while fraction D yielded **2** (15 mg) as colourless powder from chloroform, mp 274–276 °C. Later fractions (G&H) from the column gave **3** (17 mg) as colourless powder from chloroform; mp 288–290 °C. The most polar eluate was obtained from fraction I ($CHCl_3$–MeOH., 90:10) as yellow needles **4** (20 mg), which was further re-crystallized from methanol; mp 301–303 °C.

1-(6′-Hydroxy-1′,3′-benzodioxol-5′-yl)-2-(6″-hydroxy-2″-isopropenyl-2″,3″-dihydro-benzofuran-5″-yl)-ethane-1,2-dione (Calophione A) **1**; White amorphous powder; mp 120–122 °C; – ^1H and ^{13}C NMR ($CDCl_3$) spectra, see Table 1. –(+)-ESI MS: *m/z* (%) = 391 $[M+Na]^+$. –(+)-ESI HRMS: *m/z* = 369.09699 $[M+H]^+$, 369.09688 calc. for $C_{20}H_{17}O_7$.

Tephcalostan B **2**; Colourless powder; mp 274–276 °C; – ^1H and ^{13}C NMR (DMSO) spectra, see Table 2. –(+)-ESI HRMS: *m/z* = 349.07073 $[M+H]^+$, (calc. 349.07067 for $C_{20}H_{13}O_6$).

Tephcalostan C **3**; Colourless powder; mp 288–290 °C; ^1H and ^{13}C NMR (DMSO) spectra, see Table 2. ^1H and ^{13}C NMR (DMSO) spectra, see Table 2. – (−)-ESI MS: *m/z* (%) = 379.2 ($[M-H]^+$. – (+)-ESI HRMS: *m/z* = 381.0967080 $[M+H]^+$, (calc. 381.09688 for $C_{21}H_{17}O_7$).

Tephcalostan D **4**; Yellow needles; mp 301–303 °C; ^1H and ^{13}C NMR (DMSO) spectra, see Table 2. –(−)-ESI MS: *m/z* (%) = 377 ($[M-H]^+$. –(+)-ESI HRMS: *m/z* = 379.08113 $[M+H]^+$, (calc. 379.08177 for $C_{21}H_{15}O_7$).

4.4. Bioassays

4.4.1. Cytotoxicity bioassays

RAW, HT-29 cell lines were seeded at a density of 1×10^4 cells (cell viability was determined by Trypan blue exclusion dye method) (Mosmann, 1983; Wilson, 2000) per each well in 100 μl of DMEM (Dulbeccos Modified Eagles Medium) supplemented with 10% FBS (Fetal bovine serum). Twelve hours after seeding, above media was replaced with fresh DMEM supplemented with 10% FBS then 20 μl sample from above stock solutions were added to each well in triplicates which gives final concentration of 100, 50, 25, and 10 μg/well. The above cells were incubated for 48 h at 37 °C with 5% CO_2. After 48 h incubation, the above media was replaced with 100 μl of fresh DMEM with out FBS and to this 10 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (5 mg dissolved in 1 ml of FBS) was added and incubated for 3 h at 37 °C with 5% CO_2. After 3 h incubation the above media was removed with a multichannel pipette, then 200 μl of DMSO (dimethyl sulfoxide) was added to each well and the incubated at 37 °C for 15 min. Finally, the plate was read at 570 nm using spectrophotometer (Spectra-Max, Molecular devices). The IC_{50} value was defined as the concentration of sample which reduced absorbance by 50% relative to the vehicle-treated control.

4.4.2. Antiprotozoal activity

4.4.2.1. In vitro assay for Trypanosoma brucei rhodesiense. *T. brucei rhodesiense* STIB 900 strain and the standard drug melarsoprol (Arsobal) were used for the assay. Minimum essential medium (50 μL) supplemented (Baltz et al., 1985) with 2-mercaptoethanol and 15% heat-activated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were prepared covering a range from 90 to 0.123 μg/mL and then added to the wells. Then 10 bloodstream forms of *Trypanosoma b. rhodesiense* STIB 900 in 50/l were added to each well and the plate incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. Ten microliters of resazurin solution (Orhan et al., 2006; Weniger et al., 2006) (12.5 mg resazurin dissolved in 100 ml distilled water) was then added to each well and incubation continued for a further 2–4 h. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm (Raz et al., 1997) Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic programme Softmax Pro (Molecular Devices), which calculated IC_{50} values.

4.4.3. In vitro assay for Trypanosoma cruzi

Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well in 100/l RPMI1640 medium with 10% FBS and 2 mM L-glutamine. After 24 h the medium was removed and replaced by 100/l per well containing 5000 trypomastigote forms of *T. cruzi* (Tulahuen strain C2C4 containing the *p*-galactosidase (Lac Z) gene) (Weniger et al., 2006). Forty-eight hours later, the medium was removed from the wells and replaced by 100/l fresh medium with or without a serial drug dilution. Seven three-fold dilutions were used covering a range from 90 to 0.123 μg/ml). Each drug was tested

in duplicate. After 96 h of incubation, the plates were inserted under an inverted microscope to assure growth of the controls and sterility. Then the substrate CPRG/Nonidet (50 μl) was added to all wells. A colour reaction developed within 2–6 h and could be read photometrically at 540 nm. Data were transferred into the graphic programme Softmax Pro (Molecular Devices) and IC_{50} values calculated. Benznidazole was the standard drug used.

4.4.4. In vitro assay for Leishmania donovani (axenic amastigote assay)

Fifty microlitres of culture medium, a 1:1 mixture of SM medium (Cunningham, 1977) and SDM-79 medium (Brun and Schonenberger, 1979) at pH 5.4 supplemented with 10% heat-inactivated FBS, was added to each well of a 96-well microtiter plate (Costar, USA). Serial drug dilutions in duplicates were prepared covering a range from 30 to 0.041 μg/ml. Then 10 axenically grown *Leishmania donovani* amastigotes (strain MHOM-ET/67/L82) in 50/l medium were added to each well and the plate incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. Ten microliters of resazurin solution (Weniger et al., 2006) (12.5 mg resazurin dissolved in 100 ml distilled water) were added to each well and the incubation continued for a further 2–4 h. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm (Raz et al., 1997) Fluorescence development was measured and expressed as percentage of the control. Data was transferred into the graphic programme Softmax Pro (Molecular Devices) and IC_{50} values calculated. Miltefosin (Zentaris GmbH, Germany) was used as a positive reference.

4.4.5. In vitro assay for Plasmodium falciparum

Antiplasmodial activity was determined using the K1 strain of *P. falciparum* (resistant to chloroquine and pyrimethamine). A modification of the [^{3}H]-hypoxanthine incorporation assay was used (Matile and Pink, 1990). Briefly, infected human red blood cells in RPMI 1640 medium with 5% Albumax II were exposed to serial drug dilutions in microtiter plates. After 48 h of incubation at 37 °C in a reduced oxygen atmosphere, 0.5 (μCi ^{3}H-hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Betaplate TM liquid scintillation counter (Wallac, Zurich, Switzerland). The results were recorded as counts per minute (CPM), per well at each drug concentration and expressed as percentage of the untreated controls. From the sigmoidal inhibition curves IC_{50} values were calculated. The IC_{50} values are the means of four values of two independent assays carried out in duplicate.

4.4.6. Cytotoxicity

The cytotoxicity assay of the tested compounds was done following the method of Page (Page et al., 1993) with the modification of Ahmed (Ahmed et al., 1994). Cell line L-6 (rat skeletal muscle myoblasts) were seeded in 96-well Costar microtiter plates at 2×10^3 cells/100 ml, 50/l medium per well in MEM supplemented with 10% heat-inactivated FBS. A three-fold serial dilution ranging from 90 to 0.13 mg/ml of compounds in test medium was added. Plates with a final volume of 100 ml per well were incubated at 37 °C for 72 h in a humidified incubator containing 5% CO_2. Resazurin was added as viability indicator according to Ahmed et al. (1994). After an additional 2 h of incubation, the plate was measured with a fluorescence scanner using an excitation wavelength of 536 nm and an emission wavelength of 588 nm (Spectra-Max GeminiXS, Molecular Devices). Podophyllotoxin (Polysciences Inc., USA) was used as a positive reference.

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References

Ahmed, S.A., Gogal, R.M., Walsh, J.E., 1994. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H] thymidine incorporation assay. J. Immunol. Meth. 170, 211–224.

Ayafor, J.F., Tsopmo, A., Tene, M., Kamnaing, P. Ngnokam, D., Sterner, O., Iwu, M., 1997. In: Proceeding of the 7th NAPRECA Symposium on Natural Products, Dar es Salaam, Tanzania, 17–22 August, p. 99.

Baltz, T., Baltz, D., Giroud, C., Crockett, J., 1985. Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense*. EMBO J. 4, 1273–1277.

Brun, R., Schonenberger, M., 1979. Cultivation and in vitro cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Acta Trop. 36, 289–292.

Choudhary, M.I., Hareem, S., Siddiqui, H., Anjum, S., Ali, S., Atta-ur-Rahman, Zaidi, M.I., 2008. A benzil and isoflavone from *Iris tenuifolia*. Phytochemistry 69, 1880–1885.

Chien-Chang, S., Ting-Wei, L., Yu-Ling, H., Shr-Ting, W., Shien, Bor-Jinn, Chien-Chih, C., 2006. Phenolic constituents of the roots of *Sophora flavescens*. J. Nat. Prod. 69, 1237–1240.

Cunningham, I., 1977. New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. J. Protozool. 24, 325–329.

Dewick, P.M., 1993. In: Harbone, J.B. (Ed.), The Flavonoids: Advances in Research Since 1986. Chapman and Hall, London, pp. 117–238.

Ferrari, F., Alves de Lima, R., Bettolo, G.M., 1984. Flavonoids and isoflavonoids from *Zollernia paraensis*. Part 3. 2,4,2′-trihydroxy-4′-methoxybenzil from *Zollernia paraensis*. Phytochemistry 23, 2691–2692.

Ganapaty, S., Thomas, P.S., Sudheer, P., Ramana, K.V., Waterman, P.G., 2003. Flavonoids of *Tephrosia procumbens* Buch-Ham. J. Nat. Remed. 3/1, 101.

Ganapaty, S., Sreelakshmi, G.V.K., Steve, T.P., Laatsch, H., 2008. A pyranochalcone and flavanones from *Tephrosia pulcherrima* (Baker) Drumm. Nat. Prod. Commun. 3 (1), 49–52.

Kishore, P.H., Bhaskar Reddy, M.V., Gunasekar, D., Murthy, M.M., Caux, C., Bodo, B., 2003. A new coumestan from *Tephrosia calophylla*. Chem. Pharm. Bull. 51, 194–196.

Li, W., Asada, Y., Yoshikawa, T., 1998. Antimicrobial flavonoids from *Glycyrrhiza glabra* hairy root cultures. Planta Med. 64, 746–747.

Mahabusarakam, W., Deachathai, S., Phongpaichit, S., Jansakul, C., Taylor, W.C., 2004. A benzil and isoflavone derivatives from *Derris scandens* Benth. Phytochemistry 65, 1185–1191.

Miyase, T., Sano, M., Yoshino, K., Nonaka, K., 1999. Antioxidants from *Lespedeza homoloba* (I). Phytochemistry 52, 311–319.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65, 55–63.

Orhan, I., Sener, B., Atıcı, T., Brun, R., Perozzo, R., Tasdemir, D., 2006. Turkish freshwater and marine macrophyte extracts show in vitro antiprotozoal activity and inhibit FabI, a key enzyme of Plasmodium falciparum fatty acid biosynthesis. Phytomedicine 13 (6), 388–393.

Page, C., Page, M., Noel, C., 1993. A new fluorimetric assay for cytotoxicity measurements in vitro. Int. J. Oncol. 3, 473–476.

Pink, J.R.L., 1990. *Plasmodium falciparum* malaria parasite cultures and their use in immunology. In: Lefkovits, I., Pernis, B. (Eds.), Immunological Methods. Academic Press, San Diego, pp. 221–234.

Raz, B., Iten, M., Grether-Bühler, Y., Kaminsky, R., Brun, R., 1997. The Alamar blue assay to determine drug sensitivity of African trypanosomes (*T. b. rhodesiense* and) in vitro. Acta Trop. 68, 139–147.

Sauvain, M., Dedet, J., Kunesch, N., Poisson, J., 1994. Isolation of flavans from the Amazonian shrub *Faramea guianensis*. J. Nat. Prod. 57, 403–406.

Thammanna, Narayana, R.K., Chetty, M.K., 1994. Angiospermic Wealth of Tirumala. TTD Press, Tirupati. 49.

Torres-Santos, E.C., Moreira, D.L., Kaplan, M.A.C., Meirelles, M.N., Bergmann, B.R., 1999. Selective effect of 2′,6′-dihydroxy-4′-methoxychalcone isolated from *Piper aduncum* on *Leishmania amazonensis*. Antimicrob. Agent Chemother. 43, 1234–1241.

Valsaraj, R., 1997. New anti-HIV-1, antimalarial, and antifungal compounds from *Terminalia bellerica*. J. Nat. Prod. 60, 739–742.

Wang, W., Yu-Ying, Zhao, Liang, H., Qi, Jia, Hu-Biao, Chen, 2006. Coumestans from *Hedysarum multijugum*. J. Nat. Prod. 69, 876–880.

Weniger, B., Vonthron-Se necheau, C., Kaiser, M., Brun, R., Anton, R., 2006. Comparative antiplasmodial, leishmanicidal and antitrypanosomal activities of several biflavonoids. Phytomedicine 13, 176–180.

Wilson, A.P., 2000. In: Masters, J.R.W. (Ed.), Cytotoxicity and Viability Assays in Animal Cell Culture: A Practical Approach, vol. 1, third ed. Oxford University Press, Oxford, pp. 175–219.