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Research Paper

Anti-African trypanocidal and antimalarial activity of natural flavonoids, dibenzoylmethanes and synthetic analogues

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Abstract

Objectives The known anti-protozoal activity of flavonoids has stimulated the testing of other derivatives from natural and synthetic sources.
Methods As part of our efforts to find potential lead compounds, a number of flavonoids isolated from *Neoraputia paraensis, N. magnifica, Murraya paniculata*, (Rutaceae), *Lonchocarpus montanus, L. latifolius, L. subglaucescens, L. atropurpureus, L. campestris, Deguelia hatschbachii* (Leguminosae), dibenzoylmethanes from *L. subglaucescens* and synthetic analogues were tested for in-vitro activity against chloroquine-sensitive *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense* bloodstream form trypomastigotes. An assay with KB cells has been developed in order to compare in-vitro cytotoxicity of flavonoids with a selective action on the parasites.
Key findings Thirteen of the compounds tested had IC50 values ranging from 4.6 to 9.9 μM against *T. brucei rhodesiense*. In contrast, a small number of compounds showed significant activity against *P. falciparum*; seven of those tested had IC50 values ranging from 2.7 to 9.5 μM. Among the flavones only one had IC50 < 10 μM (7.6 μM), whereas against *T. brucei rhodesiense* seven had IC50 < 10 μM. Synthetic dibenzoylmethanes were the most active in terms of number (five) of compounds and the IC50 values (2.7–9.5 μM) against *P. falciparum*.
Conclusions Dibenzoylmethanes represent a novel class of compounds tested for the first time as antimalarial and trypanocidal agents.
Keywords antimalarial; flavones; flavonoids; trypanocidal agent

Introduction

The need for novel and more selective agents to treat protozoal diseases remains. The classic examples of these diseases are Chagas' disease, sleeping sickness, leishmaniasis and malaria. Chagas' disease, caused by the protozoan *Trypanosoma cruzi*, is estimated to affect some 16–18 million people, mostly from South and Central America, where 25% of the total population is at risk.[1] Besides having low efficacy, the drugs currently available, nifurtimox and benznidazole, have strong side effects.[2,3]

The bloodstream form of the parasite *T. cruzi* has no functional tricarboxylic acid cycle, and it is highly dependent on glycolysis for ATP production.[2] This great dependence on glycolysis as a source of energy makes the glycolytic enzymes attractive targets for trypanocidal drug design. The structure of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from glycosomes of *T. cruzi* (TcGAPDH) has been determined.[2] It catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. Glycosomal TcGAPDH shows potential target sites with significant differences compared with the homologous human enzyme. Thus inhibitors have been designed, synthesized, obtained from natural sources, and tested.[2] To identify powerful inhibitors, flavonoids isolated from *Neoraputia paraensis*, *N. magnifica*, *Murraya paniculata*, *Citrus sinensis* grafted on *C. limon* (Rutaceae) and *Lonchocarpus montanus* (Leguminosae) were assayed and evaluated by interaction with the enzyme TcGAPDH.[4,5] Highly oxygenated flavones have been shown to be active as GAPDH inhibitors.[4,5] However, these flavonoids were not active against *T. cruzi*

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trypomastigotes *in vitro*. This may have been due to variations in the intracellular concentration of the drug. Plant metabolites active against *T. cruzi* were extensively reviewed, showing that, in general, few flavonoids inhibited *T. cruzi* trypomastigotes *in vitro* with IC50 values higher than those for the standard therapeutic entities benznidazole and nifurtimox (less than 3 μg/ml or 10 μM).[6] Less oxygenated and methoxylated flavones have been reported as effectives in an in-vitro screen against *T. cruzi* epimastigotes. The IC50 values for 5,7,4′-trihydroxy-6-methoxyflavone (hispidulin) and 5,7-dihydroxy-3,6,4′-trimethoxyflavone were 46.7 and 47.4 μM, respectively.[7]

On the other hand, the promising results on the glycosomal enzyme TcGAPDH have stimulated us to test some flavonoids for activity against the related parasite *T. brucei rhodesiense*. African trypanosomiasis or sleeping sickness is caused by *Trypanosoma brucei rhodesiense* (East to South Africa) and *Trypanosoma brucei gambiense* (West and Central Africa). They affect approximately half a million people in sub-Saharan Africa and an estimated 60 million people are at risk of contracting disease, which is fatal if untreated.[8] The chemotherapy of these diseases is inadequate due to problems of drug resistance, variable efficacy between strains or species of the causative organisms, toxicity, difficulty of administration or requiring a long course of administration.[9]

TcGAPDH activity was only inhibited by 60–65% when chalcones were added to the assay system at a concentration of 100 μg/ml, suggesting that chalcones acted as weak inhibitors.[4] Synthetic chalcan-1,3-dione and chalcone-1,3-diol (dibenzoylmethanes) analogues showed marked loss of activity, illustrating the importance of the α,β-unsaturated ketone. However, a series of chalcones were reported as potential agents against chloroquine-resistant malaria, stimulating us to test some flavonoids for antimalarial activity.[10–12] Human malaria is responsible for considerable morbidity and mortality worldwide. It is caused by any of four species of *Plasmodium, P. vivax, P. malariae, P. ovale* and *P. falciparum*. Of these four species *P. falciparum* is responsible for the vast majority of the 300–500 million episodes of malaria worldwide and accounts for 0.7–2.7 million annual deaths. In many endemic countries, malaria is responsible for economic stagnation, lowering the annual economic growth in some regions by up to 1.5%.[13] These factors increase the urgency of the search for novel agents to treat protozoal diseases. As part of our efforts to find potential lead compounds, 11 flavones, five flavonols, four flavanones, two dihydroflavonols, four chalcones, one dihydrochalcone, two 3-phenylcoumarins, one pterocarpane, one rotenoid, two dibenzoylmethanes and synthetic derivatives such as 12 dibenzoylmethanes and one dibenzoyl were assayed against chloroquine-sensitive *P. falciparum*, *T. brucei rhodesiense in vitro* and, for cytotoxicity, against KB cells (nasopharynx carcinoma-cell culture).

Materials and Methods

Plant material, isolation and identification of compounds

All data on plant material, isolation and identification of compounds data has been published previously. In this study we cited the isolation of compounds used in the assays only (Figure 1).

Neuraputia magnifica var. *magnifica* was collected in Espirito Santo, Brazil, and a voucher specimen was deposited in the Herbarium of Instituto de Ciências Biológicas-USP-São Paulo.[4] Ground fruits were extracted with hexane, followed by CH_2Cl_2 and finally with methanol. The concentrated hexane extract after repetitive chromatographic separation afforded 2′-hydroxy-3,4,4′,5,6′-pentamethoxy-chalcone (**8**), 2′-hydroxy-3,4,4′,5-tetramethoxy-5′,6′-(2″, 2″-dimethylpyrano)-chalcone (**9a**), 2′-hydroxy-4,4′-dimethoxy-5′,6′-(2″,2″-dimethylpyrano)-chalcone (**9b**), and 2′-hydroxy-3,4,4′-trimethoxy-5′,6′-(2″,2″-dimethylpyrano)-chalcone (**9c**). The concentrated CH_2Cl_2 extract after repetitive chromatographic separation afforded, 3′,4′,5′,5,7-pentamethoxyflavanone (**5a**), 3′,4′-methylenedioxy-5,7-dimethoxyflavone (**1b**), and 3′,4′,5′,5,7-pentamethoxyflavone (**1a**).

Neoraputia paraensis was collected in Uruçuca, Bahia, Brazil, and a voucher specimen was deposited in the Herbarium of Instituto de Ciências Biológicas-USP-São Paulo.[5] Ground leaves were extracted with hexane, followed by CH_2Cl_2 and finally with MeOH. The concentrated methanol extract after repetitive chromatographic separation afforded 3′,4′,7,8-tetramethoxy-5,6-(2″,2″-dimethylpyrano)-flavone (**4**).

Murraya paniculata was collected in São Carlos, SP, Brazil, and a voucher specimen was deposited in the Herbarium of Instituto de Ciências Biológicas-USP-São Paulo.[14] The seeds, peel and pulp of the fresh ripe fruits were separated. The seeds and peel were dried, powdered and successively extracted with hexane, CH_2Cl_2 and methanol. The pulp was stirred with MeOH at room temperature. The concentrated CH_2Cl_2 extract of peel after repetitive chromatographic separation afforded 3′,4′,5′,5,6,7-hexamethoxy-flavone (**1h**), 3′,4′,5′,3,5,7,8-heptamethoxyflavonol (**1f**), 3′,4′,5′,5,7,8-hexamethoxyflavone (**1d**), 5-hydroxy-3′,4′,5′, 3,7,8-hexamethoxyflavonol (**1c**), and 8-hydroxy-3,3′,4′,5′, 5,7-hexamethoxyflavonol (**1g**). The concentrated hexane-soluble pulp fraction after repetitive chromatographic separation afforded 5-hydroxy-3′,4′,5′,3,7,8-hexamethoxyflavonol (**1c**), and 3′,4′,5′,3,5,6,7-heptamethoxyflavonol (**1e**). The concentrated CH_2Cl_2-soluble pulp fraction after repetitive chromatographic separation afforded 8-hydroxy-3,3′,4′,5′,5,7-hexamethoxyflavonol (**1g**), 3′,4′,5′,5,7,8-hexamethoxyflavone (**1d**), and 3′,4′,5′,3,5,7,8-heptamethoxyflavonol (**1f**).

Roots of *Lonchocarpus latifolius* were collected at Santa Elisa Farm of the Instituto Agrônomico de Campinas, and a voucher specimen was deposited in the Herbarium of Campinas State University, Campinas, SP, Brazil.[15,16] Dried roots were successively extracted with petrol (30–60°C), CH_2Cl_2 and methanol for 60 h in a Soxhlet apparatus. The concentrated petrol extract after repetitive chromatographic separation afforded, lanceolatin B (**2a**), karanjin (**2b**) and pongamol (**11**).

Roots of *Lonchocarpus campestris* were collected at Universidade Estadual de Campinas, Campinas, SP, Brazil, and a voucher specimen was deposited in the Herbarium of Campinas State University, Campinas, SP, Brazil.[17] Dried roots were successively extracted with petrol (30–60°C), CH_2Cl_2 and methanol for 60 h in a Soxhlet apparatus. The

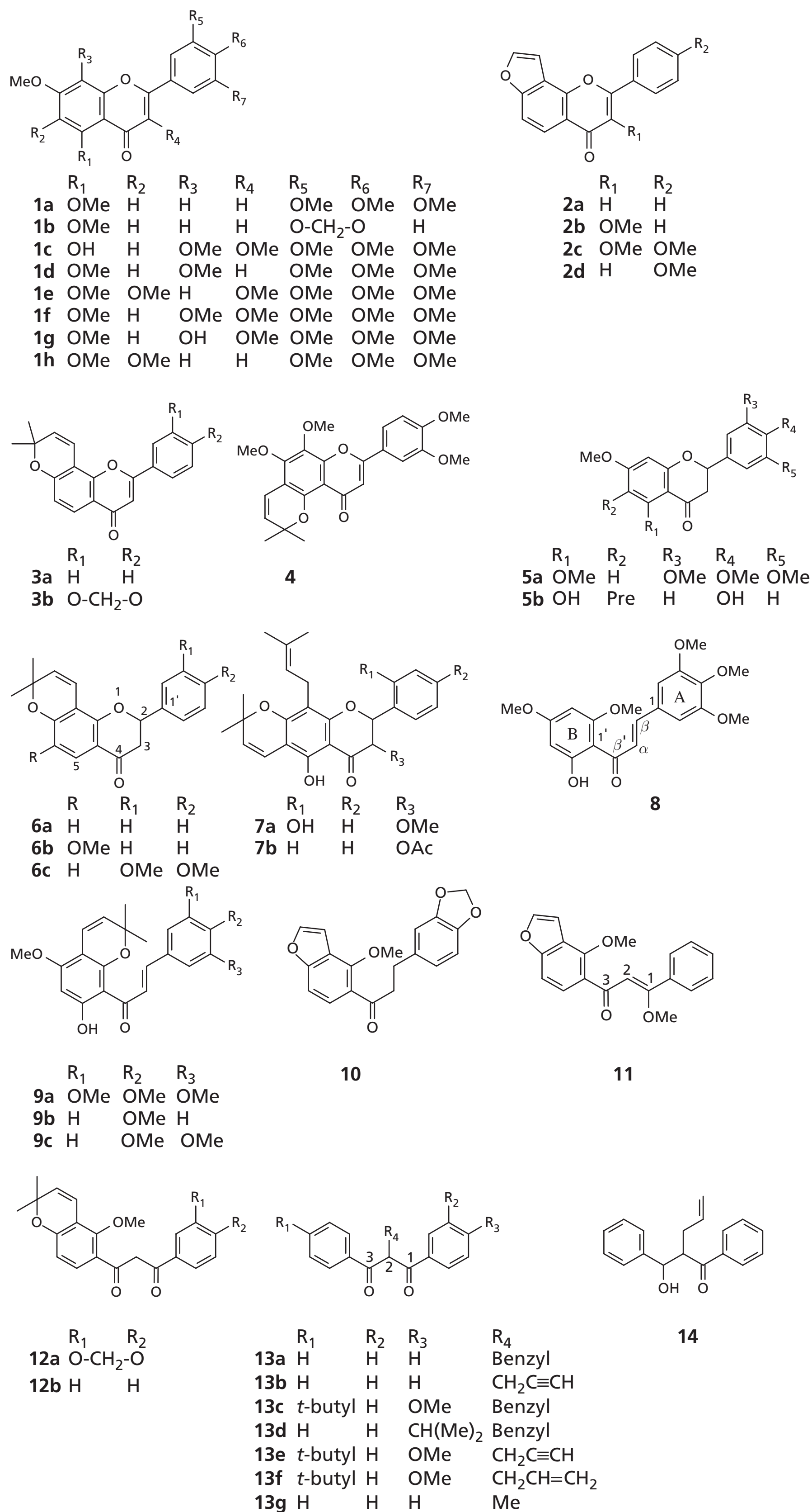

Figure 1 (*Continued*)

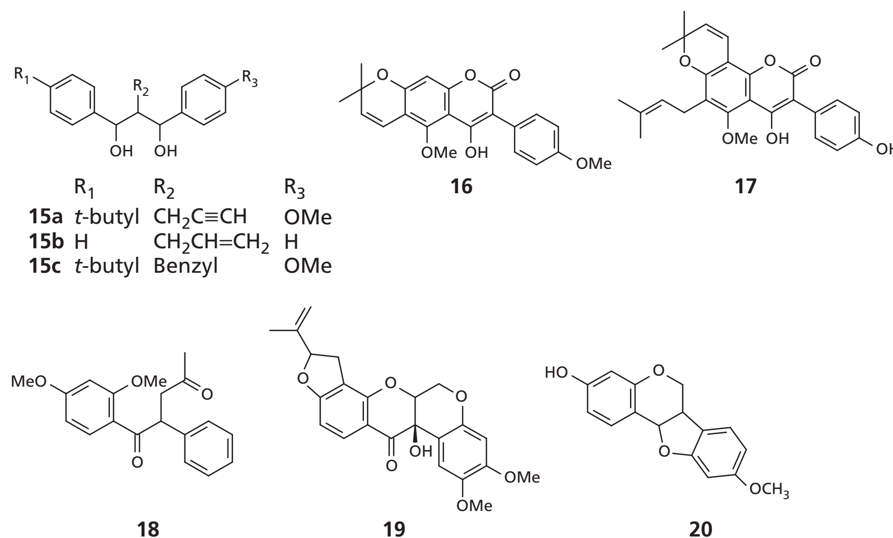

Figure 1 *(Continued)* Structures of compounds isolated for the study.

concentrated CH_2Cl_2 extract after repetitive chromatographic separation afforded, 4′-methoxykaranjin (**2c**) and isoloncho-carpin (**6a**).

Roots of *L. montanus* were collected in Taquatinga do Tocantis, Tocantis, Brazil, and a voucher specimen was deposited in the Herbarium of Campinas State University, Campinas, SP, Brazil.[18] Dried roots were successively extracted with petrol (30–60°C), CH_2Cl_2 and methanol for 60 h in a Soxhlet apparatus. The concentrated CH_2Cl_2 extract after repetitive chromatographic separation afforded 4′-methoxylanceolatin B (**2d**) and medicarpin (**20**).

Roots of *L. subglaucescens* were collected at Santa Elisa Farm of the Instituto Agrônomico de Campinas, and a voucher specimen was deposited in the Herbarium of Campinas State University, Campinas, SP, Brazil.[19] Dried roots were successively extracted with petrol (30–60°C), CH_2Cl_2 and methanol. The concentrated petrol extract after repetitive chromatographic separation afforded 7,8-(2″,2″-dimethylpyrano)-flavone (**3a**), 3′,4′-methylenedioxy-7, 8-(2″,2″-dimethylpyrano)-flavone (**3b**), 6-methoxy-6″,6″-dimethylchromeno-[2″,3″:7,8]-flavanone (**6b**), 3,4-methylenedioxy-2′-methoxy-[2″,3″:4′,3′]-furanodihydrochalcone (**10**), 3,4-methylenedioxy-2′-methoxy-6″,6″-dimethylchromeno[2″,3″:4′,3′]-β-oxochalcone (**12a**), 2′-methoxy-6″, 6″-dimethylchromeno[2″,3″:4′,3′]-β-oxochalcone (**12b**), 12-hydroxyrotenone (**19**), and 3′,4′-dimethoxy-7,8-(2″,2″-dimethylpyrano)-flavanone (**6c**).

Roots of *Deguelia hatschbachii* were collected at Universidade Estadual de Campinas, Campinas, SP, Brazil, and a voucher specimen was deposited in the Herbarium of Campinas State University, Campinas, SP, Brazil.[20,21] Dried roots were successively extracted with petrol (30–60°C), CH_2Cl_2 and methanol for 60 h in a Soxhlet apparatus. The concentrated CH_2Cl_2 extract after repetitive chromatographic separation afforded 5,4′-dihydroxy-6-(3,3-dimethylallyl)-7-methoxy-flavanone (**5b**), robustic acid (**16**), and scandenin (**17**).

Roots of *L. atropurpureus* were collected at Universidade Estadual de Campinas, Campinas, SP, Brazil, and a voucher specimen was deposited in the Herbarium of Campinas State University, Campinas, SP, Brazil.[22] Dried roots were successively extracted with petrol (30–60°C), CH_2Cl_2 and methanol for 154 h in a Soxhlet apparatus. The concentrated petrol extract after repetitive chromatographic separation afforded 2′,5-dihydroxy-3-methoxy-8-dimethylallyl-6, 7-(2″,2″-dimethyl-pyrano)-dihydroflavonol (**7a**), and mundulinol (**7b**).

Synthesis of dibenzoyl and dibenzoylmethane derivatives

Compounds **13a–g** were obtained by alkylation of dibenzoylmethane (1,3-diphenyl-propane-dione), Eusolex 8020 and Parsol 1789 using potassium carbonate in acetone. The products were characterized by high-resolution mass spectrometry (HRMS) and ^{1}H and ^{13}C NMR spectroscopy.[23] A second series of derivatives was obtained by reducing the diketones with $NaBH_4$ at –10°C in methanol producing ketol (**14**) and diols (**15a–c**).[23] Dibenzoyl compound was obtained by Friedel-Crafts acylation of 1,3-dimethoxybenzene with phenylacetyl chloride to give 2′,4′-dimethoxydeoxy-benzoin. The alkylation reaction with allyl bromide in tetrahydrofuran and NaH afforded 2′,4′-dimethoxy-2-allyldeoxybenzoin. The final step involved the oxidation with CuCl and $PdCl_2$ resulting in the formation of 2′,4′-dimethoxy-2-(propyl-2-one)-deoxybenzoin (**18**).[24]

Biological assays

Stock solutions of the compounds, plus control drugs, were prepared at a concentration of 20 μg/ml in dimethyl sulfoxide (DMSO; Sigma, Poole, UK), and diluted to appropriate concentration before assays. Values of IC50 were independently determined by making measurements for at least five inhibitor concentrations. The values represented means of at least three individual experiments. Inhibitor modalities were estimated from the collected data employing a nonparametric test.[25] Values with $P < 0.05$ were considered significant. Values of IC50 were calculated with MSxlfit (IDBS, Guildford, UK).

Plasmodium falciparum

Chloroquine-sensitive *P. falciparum* strain 3D7 was maintained in human A^+ erythocytes in RPMI 1640 medium (Sigma, Poole, UK) supplemented with Albumax II at 37°C in a 5% CO_2–air mixture. *P. falciparum* intra-erythrocytic cultures were set up as above, with 1% ring stage parasitaemia, 2.5% haematocrit, in triplicate in 100 μl medium in 96-well, flat-bottomed Microtest III tissue plates. Drugs were added in threefold dilution series and cultures incubated for a total of 48 h at 37°C in a 5% CO_2–air mixture. After 24 h, [^{3}H]hypoxanthine (0.2 mCi) was added to each well.[26,27] At the end of the assay, plates were rapidly freeze-thawed, harvested using a Tomtec Mach III cell harvester (Tomtec, Hamden, Connecticut) onto a 96-well format filtermat and Meltilex solid scintillant (both Wallac, Turku, Finland) added before reading in a Microbeta 1450 scintillation counter (Wallac, Turku, Finland) at 1 min/well.

Trypanosoma brucei rhodesiense

T. brucei rhodesiense STIB900 bloodstream form trypomastigotes were maintained in HMI-18 medium, with 15% heat-inactivated fetal calf serum (HarlanSeraLab, Crawley, UK) at 37°C in 5% CO_2–air mixture.[28] Before testing, trypomastigotes were washed and resuspended in fresh medium at a concentration 2×10^5/ml, and 100 μl of this suspension was added to the drug dilutions. The top concentration for the test compounds was 30 μg/ml. Pentamidine was included as the standard drug. Plates were incubated for 72 h at 37°C in a 5% CO_2–air mixture. At 72 h alamarBlue was added to the plates.[29] Plates were read after 4–5 h on Gemini fluorescent plate reader (Softmax Pro. 3.1.1, Molecular Devices, Wokingham, UK) at excitation/emission 530/585 nm with a filter cut-off at 550 nm.

Cytotoxicity assays

KB cells were seeded into 96-well plates at a concentration of 2×10^4/ml (100 μl/well). Drugs at 300, 30, 3 and 0.3 μg/ml were added in fresh overlay after 24 h, in triplicate at each concentration. Plates were incubated for 72 h at 37°C in a 5% CO_2–air mixture. At 72 h alamarBlue was added to the plates. Plates were read after 4–5 h on Gemini fluorescent plate reader (Softmax Pro. 3.1.1, Molecular Devices, Abingdon?, UK) excitation/emission 530/585 nm with a filter cut-off at 550 nm. IC50 values were calculated against the blanks and control samples.[30]

Results

A number of flavonoids isolated from *Neoraputia paraensis*, *N. magnifica*, *Murraya paniculata* (Rutaceae), *L. montanus*, *L. latifolius*, *L. subglaucescens*, *L. atropurpureus*, *L. campestris*, *Deguelia hatschbachii* (Leguminosae) and synthetic dibenzoylmethanes and dibenzoyl were tested for in-vitro activity against *P. falciparum* and *T. brucei rhodesiense*. An assay with KB cells was developed to compare in-vitro cytotoxicity of compounds with a selective action on the parasites (SI: selectivity index, IC50 value for KB divided by IC50 for *P. falciparum* or *T. brucei rhodesiense*). The results are summarized in Table 1 and the occurrence of flavonoids evaluated in Table 2. To facilitate discussion, IC50 values were codified as $IC50^{T,P,K}$ against *T. brucei rhodesiense*, *P. falciparum* and KB cells, respectively.

Thirteen of the flavonoids tested had values for $IC50^T$ ranging from 4.6 to 9.9 μM against *T. brucei rhodesiense*. Highly oxygenated flavones, chalcones and synthetic dibenzoylmethanes were the most active. 3′,4′,5′,5,6,7-Hexamethoxyflavone (**1h**), 2′-hydroxy-3,4,4′,5,6′-pentamethoxychalcone (**8**), and 2′-hydroxy-3,4,4′,5-tetramethoxy-5′,6′-(2″,2″-dimethylpyrano)-chalcone (**9a**) showed $IC50^T$ values of 4.8, 4.3, and 3.7 μM against *T. brucei rhodesiense*, respectively, suggesting that the presence of three methoxyl groups in the A-ring markedly affected the activity (rings were labelled A and B according to Liu *et al.*[12]). Significant activity was also found in those flavones and chalcones with a chromene ring such as **9a** and 3′,4′,7,8-tetramethoxy-5,6-(2″,2″-dimethylpyrano)-flavone (**4**, $IC50^T$ 4.4 μM). However, comparison of $IC50^T$ values for **4** and 7,8-(2″,2″-dimethylpyrano)-flavone (**3a**, $IC50^T$ 6.3 μM) showed that the presence of many methoxy groups in pyranoflavones was not a requirement for activity. Unfortunately, the above mentioned compounds exhibited a high level of cytotoxic activity. Only flavone **1h** was markedly less toxic to KB cells ($IC50^K$ > 100 μM), suggesting its selection as a candidate for in-vivo evaluation (SI 20.8).

The activity appeared to be affected by the introduction of the 3-OMe substituent into the flavone skeleton, since 3′,4′,5′,3,5,6,7-heptamethoxyflavonol (**1e**, $IC50^T$ 23.0 μM), 3′,4′,5′,3,5,7,8-heptamethoxyflavonol (**1f**, $IC50^T$ 36.4 μM) and 5-hydroxy-3′,4′,5′,3,7,8-hexamethoxyflavonol (**1c**, $IC50^T$ 14.2 μM) were less active. The only exception referred to 8-hydroxy-3,3′,4′,5′,5,7-hexamethoxyflavonol (**1g**, $IC50^T$ 7.5 μM). However, a hydroxyl group at C-8 affected the toxicity to KB cells ($IC50^K$ 32.7 μM). Trimethoxyphenyl A and B ring (3′,4′,5′,5,6,7- or 3′,4′,5′,5,7,8-hexamethoxy) flavones appeared to be the structural requirements to enhance in-vitro activity against *T. brucei rhodesiense* in comparison with 3′,4′,5′,5,7-pentamethoxyflavone (**1a**, $IC50^T$ > 80.5 μM), which was inactive. The role of the 3′,4′-methylenedioxy substituent related to activity against *T. brucei rhodesiense* was not clear, since its presence in 3′,4′-methylenedioxy-5,7-dimethoxyflavone (**1b**, $IC50^T$ 9.5 μM) increased activity and in 3′,4′-methylenedioxy-7,8-(2″,2″-dimethylpyrano)-flavone (**3b**, $IC50^T$ 45.9 μM) drastically reduced activity in comparison with **3a**.

Discussion

In a study of 132 different flavonoids, 48 were found which displayed an $IC50^T$ value below 10 μM against *T. brucei rhodesiense* bloodstream forms.[32,33] These results showed that, in general, flavonols were more trypanocidal than their flavone counterpart. Our results were contrary to such an interpretation. However, a structural feature exhibited on flavonols **1e, 1f** and **1c** was a high degree of O-methylation, while those from the literature were 3,3′,4′,5,6,7-hexahydroxyflavonol ($IC50^T$ 0.80 μM), 4′,5-dihydroxy-3,3′,6,7-tetramethoxyflavonol ($IC50^T$ 2.9 μM) and 3′,4′,5-trihydroxy-3,6,7-trimethoxyflavonol ($IC50^T$ 4.7 μM).[32]

Table 1 In-vitro activity of natural flavonoids, dibenzoylmethanes[a] and synthetic dibenzoylmethane and dibenzoyl derivatives against chloroquine-sensitive *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense*

Compound	IC50 (μM)		Selectivity index		Toxicity to KB cells IC50 (μM)
	T. brucei	*P. falciparum*	*T. brucei*	*P. falciparum*	
1a	>80.5	>80.6	nd	nd	>100
1b	9.5	7.6	>10.5	>13.2	>100
1c	14.2	47.2	5.5	1.6	77.8
1d	6.4	>74.6	>15.5	nd	>100
1e	23.0	>69.4	4.3	nd	>100
1f	36.4	20.8	>2.7	>4.8	>100
1g	7.5	37.7	4.4	0.9	32.7
1h	4.8	33.5	>20.8	>3.0	>100
2a	18.8	85.0	>5.3	>1.2	>100
2b	>100	>100	nd	nd	>100
2c	79.1	63.2	>1.3	>1.6	>100
2d	>100	92.4	nd	>1.1	>100
3a	6.3	12.9	4.1	2.0	26.0
3b	45.9	>86.1	>2.3	nd	>100
4	4.4	19.4	7.9	1.8	34.9
5a	24.1	>80.1	4.1	nd	>100
5b	11.2	39.6	1.8	0.5	20.1
6a	16.8	20.5	5.3	4.5	89.7
6b	55.4	>100	1.8	nd	>100
6c	9.3	14.5	4.8	3.1	44.3
7a	9.5	>80.1	5.5	<0.7	52.1
7b	>66.9	>66.9	nd	nd	>100
8	4.3	6.9	13.0	8.1	56.1
9a	3.7	12.3	12.3	3.7	45.6
9b	27.7	>81.8	>3.6	nd	>100
9c	31.3	66.6	>3.2	>1.5	>100
10	35.5	65.5	>2.8	>1.5	>100
11	8.9	19.9	4.2	1.9	37.1
12a	11.9	9.5	>8.4	>10.5	>100
12b	>89.2	>89.2	nd	nd	>100
13a	40.3	>90.8	>2.5	nd	>100
13b	>100	>100.0	nd	nd	>100
13c	9.9	6.7	>10.1	>14.9	>100
13d	11.9	6.4	>8.4	>15.6	>100
13e	15.9	23.4	>6.3	>4.3	>100
13f	11.7	8.6	>8.5	>11.6	>100
13g	>100	>100	nd	nd	>100
14	49.2	89.4	>2.0	1.1	>100
15a	33.5	36.6	>3.0	>2.7	>100
15b	>100	>100	nd	nd	>100
15c	4.6	2.7	4.2	7.1	19.1
16	74.8	>78.9	>1.3	nd	>100
17	15.6	49.4	>6.4	>2.0	>100
18	46.8	85.2	1.2	0.7	58.4
19	31.5	>73.0	0.009	<0.004	0.27
20	25.5	46.8	>3.9	>2.1	>100
Chloroquine		0.003			
Pentamidine	0.035				
Podophyllotoxin					0.003

[a]Occurrence of flavonoids in genera of Rutaceae and Leguminosae see Table 2. Positive control: chloroquine (IC50 3.0 nM, *P. falciparum*); pentamidine (IC50 35 nM, *T. brucei*). Toxicity was assayed against KB cells using podophyllotoxin (IC50 3.0 nM) as standard. The IC50 values are mean values from at least triplicate assays (the variation is a maximum of 20%). nd, not determined. For structures see Figure 1.

A second study with 52 flavonoids and six isoflavones showed that, in many cases, methylation was strongly disfavoured and resulted in an enormous reduction in trypanocidal activity.[34] The most noteworthy examples were 7,8-dihydroxyflavone and 7,8-dimethoxyflavone (IC50 0.068 and 6.1 μg/ml, respectively), and 7,8,3′,4′-tetrahydroxyflavone and 7,8,3′,4′-tetramethoxyflavone (IC50 0.5 and 67.2 μg/ml, respectively). Unfortunately no in-vitro data are available for polyhydroxylated flavonoids similar to those polymethoxylated flavonoids tested in our research. Thus

Table 2 Occurrence of flavonoids and dibenzoylmethanes evaluated against *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense*

Compound	Family/species
	Rutaceae
3′,4′,5′,5,7-Pentamethoxyflavone (**1a**)	*Neoraputia magnifica*[4,5]
3′,4′-Methylenedioxy-5,7-dimethoxyflavone (**1b**)	*N. magnifica*[4,5]
5-Hydroxy-3′,4′,5′,3,7,8-hexamethoxyflavonol (**1c**)	*Murraya paniculata*[14]
3′,4′,5′,5,7,8-Hexamethoxyflavone (**1d**)	*M. paniculata*[14]
3′,4′,5′,3,5,6,7-Heptamethoxyflavonol (**1e**)	*M. paniculata*[14]
3′,4′,5′,3,5,7,8-Heptamethoxyflavonol (**1f**)	*M. paniculata*[14]
8-Hydroxy-3,3′,4′,5′,5,7-hexamethoxyflavonol (**1g**)	*M. paniculata*[14]
3′,4′,5′,5,6,7-Hexamethoxyflavone (**1h**)	*M. paniculata*[14]
3′,4′,7,8-Tetramethoxy-5,6-(2″,2″-dimethylpyrano)-flavone (**4**)	*N. paraensis*[5]
3′,4′,5′,5,7-Pentamethoxyflavanone (**5a**)	*N. magnifica*[4,5]
2′-Hydroxy-3,4,4′,5,6′-pentamethoxychalcone (**8**)	*N. magnifica*[4,5]
2′-Hydroxy-3,4,4′,5-tetramethoxy-5′,6′-(2″,2″-dimethylpyrano)-chalcone (**9a**)	*N. magnífica*[4,5,31]
2′-Hydroxy-4,4′-dimethoxy-5′,6′-(2″,2″-dimethylpyrano)-chalcone (**9b**)	*N. magnifica*[4,5]
2′-Hydroxy-3,4,4′-trimethoxy-5′,6′-(2″,2″-dimethylpyrano)-chalcone (**9c**)	*N. magnifica*[4,5]
	Leguminosae
Lanceolatin B (**2a**)	*Lonchocarpus latifolius*[16]
Karanjin (**2b**)	*L. latifolius*[16]
4′-Methoxykaranjin (**2c**)	*L. campestris*[17]
4′-Methoxylanceolatin B (**2d**)	*L. montanus*[18]
7,8-(2",2"-Dimethylpyrano)-flavone (**3a**)	*L. subglaucescens*[19]
3′,4′-Methylenedioxy-7,8-(2″,2″-dimethylpyrano)-flavone (**3b**)	*L. subglaucescens*[19]
5,4′-Dihydroxy-6-(3,3-dimethylallyl)-7-methoxy-flavanone (**5b**)	*Deguelia hatschbachii*[21]
Isolonchocarpin (**6a**)	*L. campestris*[17]
6-Methoxy-6″,6″-dimethylchromeno-[2″,3″:7,8]-flavanone (**6b**)	*L. subglaucescens*[19]
3′,4′-Dimethoxy-7,8-(2″,2″-dimethylpyrano)-flavanone (**6c**)	*L. subglaucescens*[19]
2′,5-Dihydroxy-3-methoxy-8-dimethylallyl-6,7-(2″,2″-dimethylpyrano)-dihydroflavonol (**7a**)	*L. atropurpureus*[22]
3-Acetylmundulinol (natural mundulinol, **7b**)	*L. atropurpureus*[22]
3,4-Methylenedioxy-2′-methoxy-[2″,3″:4′,3′]-furanodihydrochalcone (**10**)	*L. subglaucescens*[19]
7-Methoxypongamol (natural 1-OH, **11**)	*L. latifolius*[16]
3,4-Methylenedioxy-2′-methoxy-6″,6″-dimethylchromeno[2″,3″:4′,3′]-β-oxochalcone (**12a**)	*L. subglaucescens*[19]
2′-Methoxy-6″,6″-dimethylchromeno[2″,3″:4′,3′]-β-oxochalcone (**12b**)	*L. subglaucescens*[19]
Robustic acid (**16**)	*D. hatschbachii*[20]
Scandenin (**17**)	*D. hatschbachii*[20]
(6aβ,12aβ)-12a-Hydroxy-4′,5′-tetrahydro-2,3-dimethoxy-5′-isopronenylfuran-[2′,3′:9,8]-6H-rotoxen-12-one (12-hydroxyrotenone; **19**)	*L. subglaucescens*[19]
Medicarpin (**20**)	*L. montanus*[18]

our data cannot be effectively compared with the above correlation.

Flavanones (**5**,**6**), dihydroflavonols (**7**) and dihydrochalcone (**10**) had IC50^T values ranging from 11.2 to > 66.9 μM, indicating that the structural requirement for activity against *T. brucei rhodesiense* was an α,β-unsaturated ketone in ring C. Such results were consistent with those obtained with the 132 flavonoids commented on above, which also showed that compounds lacking a double bond between C-2 and C-3 did not possess significant activity.[32] In contrast, 3′,4′-dimethoxy-7,8-(2″,2″-dimethylpyrano)-flavanone (**6c**, IC50^T 9.3 μM) and 2′,5-dihydroxy-3-methoxy-8-dimethylallyl-6,7-(2″,2″-dimethylpyrano)-dihydroflavonol (**7a**, IC50^T 9.5 μM) were potent against *T. brucei rhodesiense*. However, they were toxic to KB cells (IC50^K 44.3 and 52.1 μM, respectively), indicating no selective action on the parasites (SI 4.8 and 5.5, respectively).

The activity of synthetic 1-(4-methoxyphenyl)-2-benzyl-3-(4-*tert*butylphenyl)-1,3-propanediol (**15c**, IC50^T 4.6 μM) was comparable with that of **1h** (IC50^T 4.8 μM).[23] Five of the dibenzoylmethanes had IC50^T values ranging from 8.9 to 11.9 μM; however, the most active, **15c** and 7-methoxypongamol (**11**, IC50^T 8.9 μM), were toxic to KB cells (IC50^K 19.1 and 37.1 μM, respectively). The other four dibenzoylmethanes were, in order of their decreasing activity, 1-(4-methoxyphenyl)-2-benzyl-3-(4-*tert*butylphenyl)-1,3-propanedione (**13c**, IC50^T 9.9 μM, IC50^K > 100 μM), 1-(4-methoxyphenyl)-2-allyl-3-(4-*tert*butylphenyl)-1,3-propanedione (**13f**, IC50^T 11.7 μM, IC50^K > 100 μM), 1-(4-isopropylphenyl)-2-benzyl-3-phenyl-1,3-propanedione (**13d**, IC50^T 11.9 μM, IC50^K > 100 μM), 3,4-methylenedioxy-2′-methoxy-6″,6″-dimethylchromeno[2″,3″:4′,3′]-β-oxochalcone (**12a**, IC50^T 11.9 μM; IC50^K > 100 μM).[23] Absence of a substituent at C-2 did not result in reduced activity, as shown with **12a** in comparison with **13c**, **13d** and **13f.** However, no substitution at C-4 and C-4′ drastically reduced activity as shown with 1,3-diphenyl-2-benzyl-1,3-propanedione (**13a**, IC50^T 40.3 μM), 1,3-diphenyl-2-propargyl-1,3-propanedione (**13b**, IC50^T > 100 μM)

and 1,3-diphenyl-2-methyl-1,3-propanedione (**13 g**, $IC50^T$ > 100 μM).[23] Comparison of $IC50^K$ values of **15c** ($IC50^K$ 19.1 μM) and **13c** ($IC50^K$ > 100 μM) suggested that free hydroxyl groups at C-1 and C-3 in **15c** increased the toxicity to KB cells. However, the role of free hydroxyl groups was not clear, since 1-(4-methoxyphenyl)-2-propargyl-3-(4-*tert*butyl-phenyl)-1,3-propanediol (**15a**) and 1,3-diphenyl-2-allyl-1,3-propanediol (**15b**) showed $IC50^K$ > 100 μM.[23] Clearly, this group of dibenzoylmethanes needs further investigation, in particular in-vivo assessment, as potential trypanocidal agents.

3-Phenylcoumarins (**16**), dibenzoyl (synthetic 2′,4′-dimethoxy-2-(propyl-2-one)-deoxybenzoin (**18**)), rotenoid (**19**) and pterocarpane (**20**) showed weak activity ranging from $IC50^T$ 25.5 to 74.8 μM.[24] Only scandenin (**17**) had a moderate $IC50^T$ value of 15.6 μM.

In contrast, a small number of flavonoids showed activity against *P. falciparum in vitro*. Seven of those tested had $IC50^P$ ranging from 2.7 to 9.5 μM. Among the flavones only **1b** had $IC50^P$ < 10 μM (7.6 μM), whereas against *T. brucei rhodesiense* seven had $IC50^T$ < 10 μM. Flavanones (**5**,**6**), dihydroflavonols (**7**) and dihydrochalcone (**10**) had $IC50^P$ values ranging from 20.5 to > 100 μM, which means weak activity against *P. falciparum* as compared with *T. brucei rhodesiense in vitro*. Chalcone **8** had an $IC50^P$ value of 6.9 μM against *P. falciparum*, which was approximately equivalent to that observed with *T. brucei rhodesiense*. Comparison of the $IC50^{T,P}$ values for chalcones **8** ($IC50^T$ 4.3 μM, $IC50^P$ 6.9 μM) and **9a** ($IC50^T$ 3.7 μM, $IC50^P$ 12.3 μM) showed that the presence of a chromene ring between C-5′ and O-6′ reduced activity to *P. falciparum* but not to *T. brucei rhodesiense*. It appeared that if some specificity for one or another protozoan existed depending on the flavonoid structure. 3′,4′,5′,5,7,8-Hexamethoxyflavone (**1d**) deserves a special mention. It was more active towards *T. brucei rhodesiense* ($IC50^T$ 6.4 μM) than against *P. falciparum* ($IC50^P$ >74.6 μM), and it was not cytotoxic ($IC50^K$ > 100 μM). Flavone **1d** was more selective than **1h** ($IC50^T$ 4.8 μM, $IC50^P$ 33.5 μM, $IC50^K$ > 100 μM), but both were candidates for in-vivo evaluation against *T. brucei rhodesiense*.

Previous studies on antimalarial chalcones have emphasized the importance of ring B for activity. In particular di- or trimethoxylated ring B has been reported to be desirable.[10] Good activity seen here with **8** (dimethoxylated ring B), agreed with these findings. However, in our study the trimethoxylated ring A was shown to be also important for activity, which had not been investigated previously. Unfortunately, 3,4,5-trimethoxylated ring A derivatives were toxic to KB cells ($IC50^K$ 56.1, 45.6 μM for **8** and **9a**, respectively). Thus, other trimethoxylated ring A derivatives of chalcones should be prepared to ascertain whether antimalarial activity is retained and cytotoxic action to KB cells is reduced. Information on the antimalarial activity of over one hundred other chalcones has been published, which provided a meaningful overview of the structural requirements for activity; however, the results obtained from **8**, **9a–c** and 3,4-methylenedioxy-2′-methoxy-[2″,3″:4′,3′]-furanodihydrochalcone (**10**) were reported here for the first time.[10–12,35]

Synthetic dibenzoylmethanes were the most actives in terms of number (five) of compounds and the values of $IC50^P$ (2.7–9.5 μM) against *P. falciparum*. Compound **15c** was the most active ($IC50^P$ 2.7 μM) followed by **13d** ($IC50^P$ 6.4 μM), **13c** ($IC50^P$ 6.7 μM), **13f** ($IC50^P$ 8.6 μM), **12a** ($IC50^P$ 9.5 μM). It is not surprising in view of the fact that dibenzoylmethanes (chalcan-1,3-dione and chalcone-1,3-diol) are structurally analogous to chalcones. In general, for antimalarial activity, the steric characteristics in the bridge portion of the chalcones appeared to be important. For example, 1-(2′,4′-dimethoxyphenyl)-3-(2,4-dichlorophenyl)-2-methyl-2-propen-1-one ($IC50^P$ 6.26 μM) and 1-(2′,4′-dichlorophenyl)-3-(2,4-dimethoxyphenyl)-2-methyl-2-propen-1-one ($IC50^P$ > 14 μM), the C-2-methylderivatives of 2,4-dichloro-2′,4′-dimethoxychalcone ($IC50^P$ 0.77 μM) and 2,4-dimethoxy-2′,4′-dichlorochalcone ($IC50^P$ 3.4 μM), respectively, showed a drastic reduction in antimalarial activity.[10] However, this was not observed in our study. We observed high antimalarial activity with the related C-2-substituted (**13c**, **13d**, **13f** and **15c**) and C-2-unsubstituted (**12a**) dibenzoylmethanes. Unfortunately, when the allyl group was exchanged with the propargyl, activity against *P. falciparum* and *T. brucei rhodesiense* were decreased as seen by comparison of **13f** (C-2 = allyl, $IC50^P$ 8.6 μM; $IC50^T$ 11.7 μM) with 1-(4-methoxyphenyl)-2-propargyl-3-(4-*tert*butylphenyl)-1,3-propanedione (**13e**) (C-2 = propargyl, $IC50^P$ 23.4 μM; $IC50^T$ 15.9 μM) and **15a** (C-2 = propargyl, $IC50^P$ 36.6 μM; $IC50^T$ 33.5 μM). Furthermore, in both cases, substituents at C-4 and C-4′, diketones or diols at C-1 and C-3 were essential for activity, since **13a**, **13b**, **13g**, **15b** and 1,3-diphenyl-2-allyl-3-hydroxy-propan-1-one (**14**) were inactive.

3-Phenylcoumarins (**16**, **17**), dibenzoyl (**18**), rotenoid (**19**) and pterocarpane (**20**) showed no appreciable activity against *P. falciparum*.

The most cytotoxic compound was 12-hydroxyrotenone ($IC50^K$ 0.27 μM, **19**). However, the KB system (nasopharynx carcinoma-cell culture) measures toxicity to human cancer cells grown in cell culture and has no necessary relationship to in-vivo activity. Many compounds which are simply toxic and have no in-vivo antitumour activity are active in this system. KB activity alone is therefore not meaningful as a criterion for antitumour activity; it can therefore be a useful screen for analogues as cytotoxic agents since the testing is rapid and requires only a small amount of material.[36,37] 12-Hydroxyrotenone (**19**) showed also significant cytotoxic activity against MCF-7 (breast cancer) and A-549 (lung cancer) cells with IC50 values of 0.01 and 0.06 μg/ml, respectively.[38] In spite of a limited amount of screening having been done, particularly in the new tumour systems, there is a chance that the rotenone group might be a useful class of compounds.

Conclusions

The results indicated that the flavonoids tested *in vitro* against *P. falciparum* and *T. brucei rhodesiense* were active at concentrations in the micromolar range, while the controls chloroquine ($IC50^P$ 3.0 nM, *P. falciparum*) and pentamidine ($IC50^T$ 35 nM, *T. brucei*) were active in the nanomolar range under the same test conditions. However, if it is assumed that the IC50 required for continued investigation is < 10 μM, the

IC50 of some natural flavones and synthetic dibenzoylmethanes are meaningful. Thus, clearly flavones **1b**, **1d**, **1h** and dibenzoylmethane **13c** need further investigation as potential trypanocidal agents; flavone **1b** and dibenzoylmethanes **12a**, **13c**, **13d**, **13f** as potential antimalarial agents. Furthermore, if it is assumed that it is possible to modify the chemical structure of compounds to improve activity and selectivity, our results could help in directing the rational design of flavonoid and dibenzoylmethane derivatives as potent and effective antiprotozoal agents. All the compounds suggested above for further investigation were not toxic to KB cells ($IC50^K > 100$ μM). Finally, dibenzoylmethanes represent a novel class of compounds tested for the first time as antimalarial and trypanocidal agents.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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