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RESEARCH ARTICLE

C-Geranylated flavonoids from *Paulownia tomentosa* fruits with antimicrobial potential and synergistic activity with antibiotics

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ABSTRACT

Context C-6-Geranylated flavonoids possess promising biological activities. These substances could be a source of lead compounds for the development of therapeutics.

Objective The study was designed to evaluate their antibacterial and antileishmanial activity.

Materials and methods C-6-Geranylated flavanones were tested in micromolar concentrations against promastigote forms of *Leishmania brazilensis*, *L. donovani*, *L. infantum*, and *L. panamensis* against methicillin-resistant *Staphylococcus aureus* (MRSA); and synergistic potential with antibiotics was analyzed. IC_{50} values (after 72 h) were calculated and compared with that of miltefosine. Flow cytometry and DNA fragmentation analysis were used the mechanism of the effect. Geranylated flavanones or epigallocatechin gallate were combined with oxacillin, tetracycline, and ciprofloxacin, and the effects of these two-component combinations were evaluated. Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were established (after 24 h), the synergy was measured by the checkerboard titration technique, and the sums of the fractional inhibitory concentrations ($\sum$FICs) were computed.

Results 3′-*O*-Methyl-5′-*O*-methyldiplacone and 3′-*O*-methyldiplacone showed good antileishmanial activities (IC_{50} 8–42 μM). 3′-*O*-Methyl-5′-hydroxydiplacone activates the apoptotic death at leishmanias, the effect of 3′-*O*-methyl-5′-*O*-methyldiplacone has another mechanism. The test of the antibacterial activity showed good effects of 3′-*O*-methyldiplacol and mimulone against MRSA (MIC 2–16 μg/mL), and in six cases, the results showed synergistic effects when combined with oxacillin. Synergistic effects were also found for the combination of epigallocatechin gallate with tetracycline or oxacillin.

Conclusion This work demonstrates anti-MRSA and antileishmanial potential of geranylated flavanones and uncovers their promising synergistic activities with antibiotics. In addition, the mechanism of antileishmanial effect is proposed.

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Flavonoid; geranyl; Leishmania; MRSA; synergy

Introduction

Pathogens that have become resistant to antibiotics, represent a critical threat in many hospitals and in communities around the world, and the development of drugs that can combat such infections is urgently needed (Alanis 2005; French 2010). *Staphylococci* are the most common causes of life-threatening diseases such as hemodialysis-related bacteraemia, endocarditis, peritonitis, pyelonephritis, sepsis, and toxic shock syndrome (Casey et al. 2007). Strains of the methicillin-resistant *Staphylococcus aureus* (MRSA) are especially virulent agents. Most of them are cross-resistant to all β-lactam antibiotics, tetracyclines and macrolides or azalides, but they are generally relatively susceptible to other antibiotics (Andremont et al. 2011). Their resistance to other classes of antibiotics is however growing rapidly (Bassetti et al. 2009). One strategy for combating infectious diseases takes account of the fact that many diseases can have multiple causes and can be treated most effectively by combining natural drugs with commonly used antibiotics (Wagner & Ulrich-Merzenich 2009).

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Leishmaniasis is a severe disease most commonly spread in Africa, Asia, and Latin America, and threatening about 350 million people worldwide. The genus *Leishmania*, a protozoan of the Hemoflagellatae group, is responsible for leishmaniasis. The rather small number of drugs currently available in the clinics makes the advent of drug resistance a problem in the treatment of leishmaniasis, and a search for new antileishmanial remedies is therefore needed (Rocha et al. 2005).

Some phenolic compounds are believed to be the promising agents for the treatment of some bacterial infections. They have shown strong ability to bind to different proteins and great affinity for cell membranes (Wagner & Ulrich-Merzenich 2009). Combinations of tetracycline or β-lactam antibiotics with baicalein (5,6,7-trihydroxyflavone) exhibit synergistic effects against MRSA (Fujita et al. 2005). The synergy of combinations of another flavonoid, taxifolin-7-*O*-α-rhamnopyranoside, with ampicillin, levofloxacin, ceftazidime or azithromycin against 10 clinical isolates of MRSA has also been evaluated (An et al. 2011). Recently, epigallocatechin gallate has been studied extensively. It has been reported that it is synergistically active in combination with β-lactams, tetracycline (TTC) (Abreu et al. 2012) or oxytetracycline against various strains of methicillin and tetracycline-resistant *S. aureus* (Nový et al. 2013). Our previous research showed a promising potential of geranylated flavanones to inhibit growth of MRSA (Navrátilová et al. 2013).

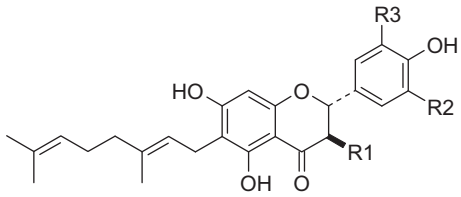
Plant products with antileishmanial activity have been reviewed by Rocha et al. (2005). More recently, some plant-derived flavonoids have been shown to exert antileishmanial activity. Quercetin has shown selective activity against leishmanial topoisomerase (Jean-Moreno et al. 2006; Cortázar et al. 2007). Some quercetin and kaempferol glycosides have been found to be active against *Leishmania* spp. (Muzitano et al. 2006). Luteolin has also been reported to induce cell death in both the promastigote and amastigote forms of *Leishmania* (Sen et al. 2006). Prenylated flavonoids isolated from *Cannabis sativa* have displayed promising activity against *L. donovani* promastigotes (Radwan et al. 2008a,b). Prenylated isoflavonoids obtained from *Psorothamnus arborescens* were found to have higher activity than their non-prenylated analogs (Salem & Werbovetz 2006). A prenylated flavonoid obtained from *Lonchocarpus* spp. affected different *Leishmania* spp. (Borges-Argáez et al. 2007). Lipophilic polymethoxylated flavonoids isolated from *Ageratum conyzoides* were active at micromolar concentrations (Nour et al. 2010).

Geranylated flavonoids seem to be promising compounds due to the presence of terpenoid chain and a phenolic part. Therefore, we tested the activity of *C*-6 geranyl flavonoids against four leishmanias: *L. brazilensis*, *L. donovani*, *L. infantum*, and *L. panamensis*, as putative antileishmanial agents, and they showed activity comparable with the standard used. Based on the previous research, we also decided to confirm the hypothesis that antibacterially active 3′-*O*-methyldiplacol (**3**) and mimulone (**5**) obtained from *Paulownia tomentosa* Thunb. Sieb. & Zucc. ex Steud. (Navrátilová et al. 2013) should show synergistic effects when combined with oxacillin (OXA), TTC or ciprofloxacin (CIP). Positive results of the screening for the antibacterial activity of geranylated flavonoids from *P. tomentosa* against different types of strains of MRSA also led us to this idea. Based on previous results, epigallocatecin gallate (EGCG, **7**) was used as a positive control and to confirm its potential to act synergically with antibiotics.

Materials and methods

Chemicals

3′-*O*-Methyl-5′-hydroxydiplacone (**1**), 3′-*O*-methyl-5′-*O*-methyldiplacone (**2**), 3′-*O*-methyldiplacol (**3**), 3′-*O*-methyldiplacone (**4**), mimulone (**5**) and diplacone (**6**) (Figure 1) were isolated from *P. tomentosa* fruits and identified as described in a previous report (Šmejkal et al. 2008). A voucher specimen (PT-04O) has been deposited at the herbarium of the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno (UVPS Brno), Brno, Czech Republic. The compounds are saved in the compound library of the department. The purity of isolated compounds exceeded 95% according to HPLC analysis. Compounds **1–6** and epigallocatechin gallate (EGCG, **7**, Sigma-Aldrich, Praha, Česká republika) were dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Praha, Česká republika) for antibacterial assays. For antileishmanial assays, compounds **1–6** were dissolved in 40% DMSO with 10% 0.1 M NaOH and used to prepare stock solutions of concentrations 0.025, 0.25, 0.625, 1.25, 2.5, 5, and 12.5 mM. In our experiments, the final concentration of DMSO in the culture medium never exceeded 0.2%, which did not affect the viability of the parasitic cells as was confirmed by cultivation with DMSO only. Deionised water and 96% ethanol were used to dissolve the standard antibiotics oxacillin (OXA), tetracycline (TTC) and ciprofloxacin (CIP) (Sigma-Aldrich, Praha, Česká republika). Bacteria were grown in the Mueller–Hinton broth (MHB) and Mueller–Hinton agar (MHA) (Oxoid, Hampshire, UK). MHB was used for susceptibility testing. MBCs were determined using MHA plates.



	R1	R2	R3
1	H	OMe	OH
2	H	OMe	OMe
3	OH	OMe	H
4	H	OMe	H
5	H	H	H
6	H	OH	H

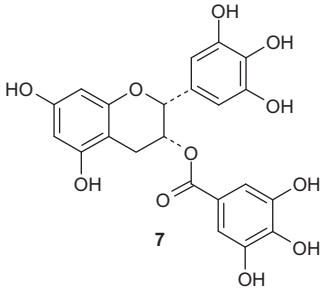


Figure 1. Structures of C-geranylated flavanones (**1**–**6**) from *P. tomentosa* fruits and epigallocatechin gallate (**7**) used for bio-activity testing.

Bacterial strains

Eleven strains of staphylococcus were used for the tests. *Staphylococcus intermedius* MRSI 82110 was obtained during surgery on a dog (on deposit at the Department of Infectious Diseases and Microbiology, UVPS Brno). Four methicillin-resistant strains of *S. aureus*: MRSA 67755, MRSA 63718, MRSA 62097, and MRSA 62059 are deposited at the Department of Infectious Diseases and Microbiology, UVPS Brno; MRSA 1098, MRSA 1679, and MRSA 1903 were obtained from the National Institute of Public Health Brno. MRSA strains, MRSA 630, MRSA 3202, and MRSA 6975 are clinical isolates deposited at the National Reference Laboratory for Antibiotics, National Institute of Public Health Prague.

Parasite cultivation

The slightly modified method of Varela-M et al. (2012) was used for the cultivation and further work with leishmanias. Promastigotes of four species, *L. braziliensis* (MHOM/CO/88/UA301), *L. donovani* (MHOM/IN/80/DD8), *L. infantum* (MCAN/ES/96/BCN150), and *L. panamensis* (MHOM/CO/87/UA140), were continually cultured at 27 °C by shaking in Schneier's medium (Sigma-Aldrich, Madrid, Spain) supplemented with $NaHCO_3$ (0.4 g/L), $CaCl_2$ (0.6 g/L), 10% fetal bovine serum (Sigma-Aldrich, Spain), and penicillin 10 000 U/mL–streptomycin 10 000 µg/mL (Sigma-Aldrich, Madrid, Spain). Parasites were transferred to a fresh medium every 2–4 d; their viability was checked periodically under a microscope. Parasites in the exponential growth phase were centrifuged at 2500 rpm for 10 min, re-suspended in a fresh medium and divided onto 96-well test plates in the amount of 10^5 per 200 µL with DMSO as a control or with the tested compound at final concentrations from 0.25 to 125 µM, incubated for 72 h at 27 °C with shaking, and analysed by XTT assay, or incubated for 24 or 48 h under the same conditions in the amount of 2×10^6 cells/mL for flow cytometry evaluation.

Cytotoxicity assay

Prior to testing of the selected *C*-geranylated flavanones on leishmania promastigotes, their effect against native rat alveolar macrophages using the MTT method was tested (Nešuta et al. 2011).

XTT assay

The cell viability was evaluated by measuring the activity of mitochondrial dehydrogenase. XTT reagent (50 µL) activated with PMS (at a ratio 49:1, v/v) was added and incubated at 27 °C for 4 or 12 h and the absorbance of the coloured formazan formed was measured at 490 nm (with 655 nm as a reference). The IC_{50} value was then calculated. All samples were measured in independent triplicates.

Flow cytometry measurement

For the analysis of a cell cycle, we used flow cytometry. Promastigote cells of *L. donovani* treated for 24 or 48 h with compounds **1** and **2** at concentrations of 12.5 and 25 µM, respectively, were centrifuged and denatured with ethanol. RNAse was added, and propidium iodide was used as a fluorescent marker of DNA. After 1 h incubation in darkness at laboratory temperature, the fragmentations of the nuclei were measured on a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon laser, using CELLQUEST software. The received data were analysed using WinMDI 2.8 (Biosciences PharMingen, San Diego, CA) and expressed as a percentage of cells in apoptosis.

Susceptibility testing

MIC and MBC values were determined by the broth microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2009), using 96-well microtiter plates. Bacterial suspension was prepared from 24 h old bacterial cultures and adjusted to 0.5 McFarland standard using Densi-La-Meter (Lachema, Brno-Řečkovice, Česká republika), and subsequently diluted with sterile distilled water to get the final concentration of 5×10^6 CFU/mL. Serial two-fold dilutions of the compounds tested were inoculated with a suspension of bacteria and incubated at 37 °C for 24 h. MIC values were defined as the lowest concentrations of an antimicrobial substance that inhibited the visible growth of a micro-organism. MBC values were determined by transferring 10 µL aliquots from wells without visible signs of growth into MHA plates and incubated at 37 °C for a further 24 h. MBC values were the lowest concentrations of an antibacterial agent resulting in the growth of not more than two CFU. All tests were carried out in duplicate in each of the two independent experiments. MIC and MBC values were calculated as an average from all tests (data not shown.)

Synergy testing

Potential anti-MRSA synergistic effects of compounds **3** and **5** with OXA, TTC, and CIP were measured by the checkerboard titration technique, and the fractional inhibitory concentrations (FICs) were evaluated according to the guidelines set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2000). The FIC results were interpreted as follows: $\sum FIC \leq 0.5 =$ synergy; $\sum FIC > 0.5 \leq 1 =$ additivity; $\sum FIC > 1$ to $< 2 =$ indifference; $\sum FIC \geq 2 =$ antagonism.

Results and discussion

The *C*-6 geranylated compounds 3′-*O*-methyl-5′-hydroxydiplacone (**1**), 3′-*O*-methyl-5′-*O*-methyldiplacone (**2**), 3′-*O*-methyldiplacol (**3**), 3′-*O*-methyldiplacone (**4**), mimulone (**5**), and diplacone (**6**), isolated from *P. tomentosa* as described previously (Šmejkal et al. 2008), were tested for their ability to kill the promastigote forms of four *Leishmania* species: *L. braziliensis*, *L. donovani*, *L. infantum* and *L. panamensis*. Prior to testing, the cytotoxicity of compounds **1**–**6** was evaluated using alveolar rat macrophages (Nešuta et al. 2011). Compounds tested showed no substantial toxicity at concentrations tested. By using XTT assays, we found that compounds **2** and **4** achieved significant antileishmanial activity, showing IC_{50} values against *L. donovani* and *L. braziliensis* comparable with that of miltefosine, which was used as a positive control (Table 1). These compounds also showed notable activity against *L. infantum* and *L. panamensis*, but at higher concentrations than miltefosine. As can be seen, the above-mentioned compounds (**2** and **4**) showed some structure–activity relation specifity in their toxic effect against certain *Leishmania* species. The most active compounds tested here were 3′-*O*-methyl substituted at the flavanone ring B. The activity of 3′-*O*-methyl substituted dihydroflavonol **3** was diminished probably due to the presence of 3-OH substitution.

Table 1. *Leishmania* species were treated 72 h with concentrations ranging from 0.25 to 125 µM of compounds **1**–**6** obtained from *Paulownia tomentosa* and with miltefosine as a control.

	Parasites			
Compound	*L. infantum*	*L. panamensis*	*L. donovani*	*L. brasilensis*
1	42.3 ± 3.7	20.7 ± 2.6	21.3 ± 4.8	13.2 ± 2.3
2	23.8 ± 3.8	21.8 ± 7.3	12.7 ± 0.7	8.0 ± 0.4
3	65.3 ± 17.3	76.7 ± 3.3	54.0 ± 9.6	27.0 ± 5.0
4	39.3 ± 23.7	24.0 ± 4.6	10.4 ± 2.4	11.3 ± 0.3
5	48.7 ± 9.9	47.7 ± 8.4	52.5 ± 12.8	54.3 ± 3.9
6	74.3 ± 38.6	72.3 ± 26.1	77.3 ± 8.7	75.0 ± 10.8
Miltefosine	19.5 ± 7.5	5.9 ± 1.2	9.5 ± 0.2	6.7 ± 2.3

The absorbance from XTT assay was measured and inhibition concentrations IC_{50} (µM) were calculated for every compound and parasite species. The values are expressed as a mean ± SEM. Values are calculated from triplicates.

C-6-Substituted compounds isolated from *C. sativa* such as cannflavin C are structurally very similar to compound **4** (except for the double bond between *C*-2 and *C*-3), and they have been reported to show an IC_{50} value of 4.5 µg/mL (cannflavin C, close to 10 µM) against *L. donovani* (Radwan et al. 2008a). Antileishmanial activity for cannflavin B (the double bond between *C*-2 and *C*-3, *C*-6 prenyl, 5 µM) has also been observed (Radwan et al. 2008b). Radwan et al. (2008a) also reported a moderate activity of cannflavin A (17 µg/mL, 39 µM). Salem et al. (2011) have published some data about the antileishmanial activity of the *C*-geranyl flavonoids that are in good agreement with the data presented here. Thus, we suggest that the presence of a prenyl side chain at *C*-6 could be an important structural feature for antileishmanial activity. For example, highly methoxylated lipophylic flavonoids obtained from *Ageratum conyzoides* have shown an effect against *L. donovani* (Nour et al. 2010). As could be deduced from the literature and our results, a higher degree of lipophility and better entrance to the parasite cell could play important roles.

For the next stage of the evaluation of the cell cycle, compounds **1** and **2** were chosen based on the XTT assay on *L. donovani*. The analysis of flow cytometry experimental data brought us a result showing a high

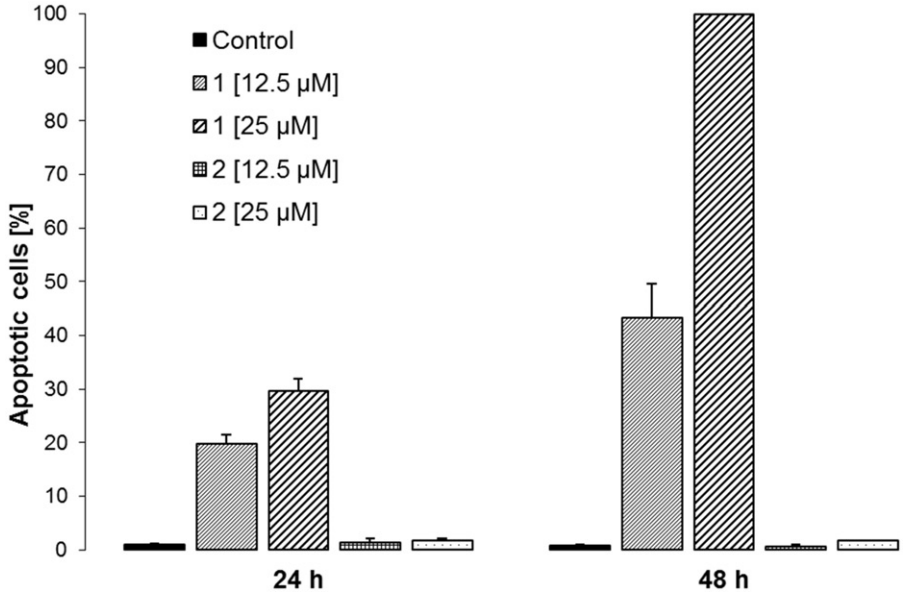


Figure 2. Apoptogenic effect of compounds **1** and **2** on *L. donovani* promastogote cells. Percentage of cells in apoptosis calculated from flow cytometry data. Experiments were run in independent duplicates.

percentage of DNA fragmentation after treatment of the parasitic cells with 3′-*O*-methyl-5′-hydroxydiplacone (**1**), which hints at the probable mechanism of effect by induction of apoptotic death (Figure 2). On the other hand, almost no DNA fragmentation after treatment of *L. donovani* promastigote cells with 3′-*O*-methyl-5′-*O*-methyldiplacone (**2**) has been observed. Comparing these results with the results obtained by XTT assay, it is obvious that the death of parasitic cells is caused in a non-apoptic way in the case of 3′-*O*-methyl-5′-*O*-methyldiplacone (**2**) and that the presence of the additional methoxy group at the ring B of the flavanone significantly changes the mechanism of action. Galzi et al. (2010) showed a connection between anti-inflammatory activity and the antileishmanial effect. The programmed cell death is closely connected to the protozoan mechanism of killing action (Bruchhaus et al. 2007). Our other experiments testing the anti-inflammatory activity of *C*-geranylated flavanones showed their ability to affect gene expression and protein synthesis in cellular model (Hošek et al. 2010) and an effect on the cell-cycle mediated by changes in the cyclins (Kollár et al. 2011), which confirms the ability of compounds **1** and **2** affect cell cycle and which support the connection of anti-inflammatory activity and the antileishmanial effect.

In our previous research, the antibacterial activity of different geranylated flavonoids against five MRSA strains (287, 4211, 6975, 630, and 62059) was shown (Navrátilová et al. 2013), where the most active compound **5** showed MIC values ranging from 2 to 4 μg/mL (4.9–8.8 μM), followed by compound **3** with MIC values ranging from 4 to 8 μg/mL (8.8–17.6 μM) towards all strains tested. To support the results of our research, MIC values against the other six different strains of MRSA for compounds **1**–**6** were screened and compounds **3** and **5** were confirmed as strongly active (Table 2). All the MRSA and MRSI strains used in assays have been considered as to be resistant to TTC with MICs ≥ 16 μg/mL (≥36 μM) (EUCAST 2000). Compounds **3**, **5,** and **7** in binary combinations with the standard antibiotics OXA, TTC, and CIP were tested for their antimicrobial activities against several methicillin-resistant strains of *S. aureus*. The results of the MIC and MBC assays for compounds **3**, **5,** and **7** are summarized in Tables 3–6.

Table 2. MIC values (μg/mL/μM) of **1**–**6**.

Compound	MRSA 1903	MRSA 63718	MRSA 3202	MRSA 62097	MRSA 67755	MRSA 1679
1	8/17.6	32/70.5	4/8.8	4/8.8	8/17.6	8/17.6
2	8/17.1	>64/>136.7	4/8.5	4/8.5	8/17.1	16/34.3
3	4/8.8	8/17.6	4/8.8	8/17.6	8/17.6	4/8.8
4	8/18.2	32/73.1	4/9.1	4/9.1	8/18.2	8/18.2
5	2/4.9	16/39.2	2/4.9	4/9.8	4/9.8	4/9.8
6	8/18.9	64/150.9	8/18.9	16/37.7	16/37.7	16/37.7
OXA	1/2.5	4/9.9	>32/79.8	1/2.5	1/2.5	1/2.5
TTC	>32/>72.1	>32/>72.1	16/36	32/72.1	16/36	32/72.1
CIP	16/48.3	8/24.2	16/48.3	1/3	0.5/1.5	1/3

MRSA, methicillin-resistant *S. aureus*; OXA, oxacillin; TTC, tetracycline; CIP, ciprofloxacin; MIC, minimum inhibitory concentration.

Table 3 shows the values obtained from the testing of **3** and **5**, with the β-lactam antibiotic OXA. The individual values of MIC or MBC for each agent against different strains of staphylococcus are displayed alone with the values of the FICs, the ∑FICs and the effect resulting

Table 3. MIC and MBC values (μg/mL) of compound **3** or **5**, ∑FIC and resultant effect of combination with OXA against various strains of MRSA and MRSI.

		MIC/MBC				
Strain	Agent	Alone	Combination	FIC	∑FIC	Result
MRSI 82110	**3**	4/4	2/4	0.5/1	0.516/2	ADD/ANT
	OXA	32/32	0.5/32	0.016/1		
MRSI 82110	**5**	2/4	0.5/0.5	0.25/0.125	0.375/0.188	SYN/SYN
	OXA	16/32	2/2	0.125/0.063		
MRSA1903	**3**	4/4	2/2	0.5/0.5	0.625/0.625	ADD/ADD
	OXA	8/8	1/1	0.125/0.125		
MRSA1903	**5**	2/2	0.031/2	0.016/1	0.266/1.5	SYN/IND
	OXA	16/16	4/8	0.25/0.5		
MRSA63718	**5**	8/8	8/16	1/2	1.031/2.063	IND/ANT
	OXA	64/64	2/4	0.031/0.063		
MRSA6975	**3**	4/4	1/2	0.25/0.5	0.313/0.75	SYN/ADD
	OXA	512/512	32/128	0.063/0.25		
MRSA6975	**5**	4/4	2/4	0.5/1	0.531/1.25	ADD/IND
	OXA	512/512	16/128	0.031/0.25		
MRSA3202	**3**	4/4	1/2	0.25/0.5	0.375/1	SYN/ADD
	OXA	256/256	32/128	0.125/0.5		
MRSA3202	**5**	4/4	1/2	0.25/0.5	0.5/1.5	SYN/IND
	OXA	256/256	64/256	0.25/1		
MRSA62097	**3**	4/4	0.031/0.031	0.008/0.008	1.008/1.008	IND/IND
	OXA	4/4	4/4	1/1		
MRSA62097	**5**	4/4	2/4	0.5/1	0.563/3	ADD/ANT
	OXA	1/2	0.063/4	0.063/2		
MRSA62059	**3**	4/4	0.031/4	0.008/1	1.008/2	IND/ANT
	OXA	4/8	4/8	1/1		
MRSA62059	**5**	4/4	0.031/0.031	0.008/0.008	1.008/1.008	IND/IND
	OXA	2/2	2/2	1/1		
MRSA67755	**3**	4/4	0.031/4	0.008/1	0.508/2	ADD/ANT
	OXA	4/4	2/4	0.5/1		
MRSA67755	**5**	4/4	0.031/4	0.008/1	1.008/2	IND/ANT
	OXA	4/8	4/8	1/1		
MRSA1679	**3**	4/4	0.063/4	0.016/1	0.266/1.5	SYN/IND
	OXA	8/8	2/4	0.25/0.5		
MRSA1679	**5**	4/4	2/2	0.5/0.5	0.625/0.563	ADD/ADD
	OXA	2/4	0.25/0.25	0.125/0.063		
MRSA630	**3**	4/4	2/2	0.5/0.5	0.625/0.563	ADD/ADD
	OXA	16/32	2/2	0.125/0.063		
MRSA630	**5**	4/4	2/2	0.5/0.5	0.625/0.625	ADD/ADD
	OXA	16/16	2/2	0.125/0.125		
MRSA1098	**3**	4/4	0.031/4	0.008/1	1.008/2	IND/ANT
	OXA	16/64	16/64	1/1		
MRSA1098	**5**	4/4	0.031/4	0.008/1	1.008/2	IND/ANT
	OXA	8/16	8/16	1/1		

MRSA, methicillin-resistant *S. aureus*; OXA, oxacillin; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; FIC, fractional inhibitory concentration; ∑FIC, sum of fractional inhibitory concentrations; SYN, synergy; ADD, additivity; IND, indifference; ANT, antagonism.

from the agent or combination of agents. In six cases, our results showed overall synergistic effects (∑FIC 0.266–0.5) for combinations of flavonoids **3** or **5** with OXA. Compound **5** combined with OXA demonstrated synergy: against MRSI 82110 (∑FIC = 0.375, MIC 8-fold reduction), MRSA 1903 (∑FIC = 0.266, MIC 4-fold reduction) and MRSA 3202 (∑FIC = 0.5, MIC 4-fold reduction). A synergistic effect of compound **3** combined with OXA against three strains of MRSA was also observed: MRSA 6975 (∑FIC = 0.313, MIC 16-fold reduction); MRSA 3202 (∑FIC = 0.375, MIC 8-fold reduction); and MRSA 1679 (∑FIC = 0.266, MIC 4-fold reduction). Additive effects were found for combinations of compound **5** and OXA against four strains of MRSA: 6975, 62097, 1679, and 630 (∑FIC 0.531–0.625). The values obtained for the additivity effects observed for compound **3** combined with OXA against MRSI 82110, MRSA 1903, MRSA 67755, and MRSA 630 (∑FIC 0.508–0.625) were barely above the upper limit for synergy. Based on the publication of Eumkeb et al. (2012), we postulated three hypothetical mechanisms for the antibacterial action of flavonoids **3** and **5** and their possible mechanism of synergy: (1) inhibition of the synthesis of the peptidoglycan layer of the bacterial cell wall, (2) alteration of the permeability of the cell wall, and (3) inhibition of β-lactamase. We also suggest that more than one mechanism of synergistic action is possible. The obvious example is baicalein (5,6,7-trihydroxyflavone), which exhibits synergistic effects with tetracycline and β-lactam antibiotics against

Table 4. MIC and MBC values (μg/mL) of compound **3** or **5**, ∑FIC and resultant effect of combination with TTC against various strains of MRSA and MRSI.

Strain	Agent	MIC/MBC		FIC	∑FIC	Result
		Alone	Combination			
SI 82110	**3**	4/4	2/2	0.5/0.5	0.75/0.625	ADD/ADD
	TTC	64/128	16/16	0.25/0.125		
SI 82110	**5**	4/4	2/4	0.5/1	0.563/1.25	ADD/IND
	TTC	64/128	4/32	0.063/0.25		
MRSA1903	**3**	4/4	1/4	0.25/1	0.75/2	ADD/ANT
	TTC	16/32	8/32	0.5/1		
MRSA1903	**5**	8/8	4/8	0.5/1	0.625/1.25	ADD/IND
	TTC	16/32	4/8	0.125/0.25		
MRSA63718	**3**	8/8	4/4	0.5/0.5	1/1	ADD/ADD
	TTC	64/64	32/32	0.5/0.5		
MRSA6975	**3**	4/4	2/2	0.5/0.50.063/0.25	0.563/0.75	ADD/ADD
	TTC	16/16	1/4			
MRSA6975	**5**	2/4	1/1	0.5/0.25	1/0.75	ADD/ADD
	TTC	16/16	8/8	0.5/0.5		
MRSA3202	**3**	4/4	2/4	0.5/1	1/3	ADD/ANT
	TTC	32/32	16/64	0.5/2		
MRSA3202	**5**	2/4	1/1	0.5/0.25	1/0.75	ADD/ADD
	TTC	32/32	16/16	0.5/0.5		

MRSA, methicillin-resistant *S. aureus*; TTC, tetracycline; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; FIC, fractional inhibitory concentration; ∑FIC, sum of fractional inhibitory concentrations; ADD, additivity; IND, indifference; ANT, antagonism.

Table 5. MIC and MBC values (μg/mL) of compound **3** or **5**, ∑FIC and resultant effect of combination with CIP against various strains of MRSA.

Strain	Agent	MIC/MBC		FIC	∑FIC	Result
		Alone	Combination			
MRSA1903	**3**	4/8	4/4	1/0.5	1.5/1.5	IND/IND
	CIP	2/16	1/16	0.5/1		
MRSA1903	**5**	4/4	4/16	1/4	1.5/4.5	IND/ANT
	CIP	2/32	1/16	0.5/0.5		
MRSA6975	**3**	2/4	0.25/2	0.125/0.5	0.625/1	ADD/ADD
	CIP	64/256	32/128	0.5/0.5		
MRSA63718	**5**	8/8	8/8	1/1	1.5/1.25	IND/IND
	CIP	8/16	4/4	0.5/0.25		
MRSA6975	**3**	4/4	2/2	0.5/0.5	1/0.625	IND/ADD
	CIP	64/256	32/32	0.5/0.125		
MRSA3202	**3**	4/4	2/2	0.5/0.5	1.5/1.5	IND/IND
	CIP	16/16	16/16	1/1		
MRSA3202	**5**	4/8	2/8	0.5/1	0.75/1.5	ADD/IND
	CIP	16/16	4/8	0.25/0.5		

MRSA, methicillin-resistant *S. aureus*; CIP, ciprofloxacin; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; FIC, fractional inhibitory concentration; ∑FIC, sum of fractional inhibitory concentrations; ADD, additivity; IND, indifference; ANT, antagonism.

MRSA (Fujita et al. 2005). The mechanism of its action is caused probably by the inhibition of efflux pumps and PBP2a or with the interaction with membrane peptidoglycan (Wagner & Ulrich-Merzenich 2009; Chan et al. 2011).

Several recent studies have reported synergistic antibacterial effects for combinations of flavonoids with β-lactams or cephalosporins, including effect on MRSA (Eumkeb et al. 2010; An et al. 2011; Eumkeb et al. 2012; Eumkeb & Chukrathok 2013). Synergistic and additive effects have also been observed for some prenylated or geranylated flavonoids. In combination with mupirocin, bidwillon B, a prenylated isoflavanone isolated from *Erythrina variegata* L. Merr. has been tested against 12 strains of MRSA, and in 11 strains, it showed synergy or additivity, acting by inhibiting the incorporation of thymidine, uridine, glucose, and isoleucine, into the cells of MRSA (Sato et al. 2004). Binary combinations of sophoraflavanone G, a geranylated flavanone isolated from *Sophora* spp., with vancomycin, fosfomycin, methicillin, cefzonam, gentamicin, minocycline, or levofloxacin, have inhibited 27 strains of MRSA. An increase in the inhibitory activity of vancomycin during the second stage of the synthesis of the bacterial cell wall has been postulated as a mechanism fostering the synergy that results from combining it with sophoraflavanone G (Sakagami et al. 1998).

The effects of combining compound **3** or **5** with TTC were designated as additive (∑FIC 0.563–1) for five strains of staphylococcus (four of them resistant to methicillin) as shown in Table 4. Synergistic effects have been observed for combinations of baicalein with TTC or CIP. It has been suggested that some strains of MRSA were inhibited by blocking the Tet(K) efflux system. Previously obtained results showed clearly that not only the inhibition of efflux but also interference with PBP or inhibition of an enzyme such as methyltransferase might contribute to the mechanism of action (Chan et al. 2011). Unfortunately, we found no significant synergy for combinations of compound **3** or **5** with CIP as shown in Table 4, where MRSA 3202 (∑FIC 0.625) and MRSA 6975 (∑FIC 0.75) were the most sensitive bacterial strains.

Table 6. MIC and MBC values (μg/mL) of compound **7**, ∑FIC and resultant effect of combination with TTC or OXA against various strains of MRSA.

		MIC/MBC				
Strain	Agent	Alone	Combination	FIC	∑FIC	Result
MRSA1903	**7**	128/128	64/128	0.5/1	0.75/1.125	ADD/IND
	TTC	64/256	16/32	0.25/0.125		
MRSA63718	**7**	64/64	32/32	0.5/0.5	1/1	ADD/ADD
	TTC	64/64	32/32	0.5/0.5		
MRSA6975	**7**	64/64	16/16	0.25/0.25	0.375/0.375	SYN/SYN
	TTC	256/256	32/32	0.125/0.125		
MRSA3202	**7**	64/64	16/16	0.25/0.25	0.5/0.5	SYN/SYN
	OXA	256/256	64/64	0.25/0.25		
MRSA62097	**7**	128/128	8/8	0.063/0.063	0.563/0.563	ADD/ADD
	OXA	1/1	0.5/0.5	0.5/0.5		
MRSA62059	**7**	128/128	4/8	0.031/0.063	0.531/1.063	ADD/IND
	OXA	1/1	0.5/1	0.5/1		
MRSA67755	**7**	128/128	32/32	0.25/0.25	0.75/0.75	ADD/ADD
	OXA	0.5/0.5	0.25/0.25	0.5/0.5		
MRSA1679	**7**	256/256	8/8	0.031/0.031	0.531/0.531	ADD/ADD
	OXA	1/1	0.5/0.5	0.5/0.5		

MRSA, methicillin-resistant *S. aureus*; compound **7**, epigallocatechin gallate; OXA, oxacillin; TTC, tetracycline; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; FIC, fractional inhibitory concentration; ∑FIC, sum of fractional inhibitory concentrations; SYN, synergy; ADD, additivity; IND, indifference.

Synergy between compound **7** and antibiotics has been shown in many cases, especially for the β-lactams benzylpenicillin, oxacillin, methicillin, ampicillin, and cefalexin (Zhao et al. 2001); the tetracyclines (Sudano Roccaro et al. 2004); and the macrolids (Kurinčič et al. 2012). Mechanisms for the antibacterial action of compound **7** are thought to be similar to those previously described for compounds **3** and **5**. In particular, the disruption of the integrity of the cell wall has been clearly established using MHB supplemented with external peptidoglycan and compound **7**. When linked to peptidoglycan, EGCG (**7**) loses its antibacterial activity (Zhao et al. 2001). Experiments with strains of MRSA containing efflux pumps have shown compound **7** to be a promising agent for inhibiting Tet(K) and Tet(B) efflux systems (Rodríguez-Rojas et al. 2013). As Table 6 shows, a synergistic effect was found in two cases: compound **7** combined with TTC and used against MRSA 6975 (∑FIC 0.375) and compound **7** combined with OXA and used against MRSA 3202 (∑FIC 0.5). The other results showed additivity (∑FIC 0.531-1) for the strains of MRSA 1903, MRSA 63718, MRSA 62097, MRSA 62059, MRSA 67755, and MRSA 1679.

The present work confirms the potential of geranylated phenolic compounds for the use in controlling the growth of antibiotic-resistant micro-organisms and several *Leishmania* species. Geranylated flavonoids were effective in lowering the MIC values of the antibiotics OXA and TTC tested on several MRSA strains. The results show the presence of the unmodified geranyl side chain as a structural fragment important for activity. The most active compound **5** possesses the 3-OH hydroxyl group. Compounds **3** and **5** alone, or combined with antibiotics, have demonstrated antibacterial activity *in vitro* that is potentially useful in combinatory therapy targeted at MRSA infections. According to our best knowledge, this is the first report of the possible synergistic effects of compounds **3** and **5** combined with oxacillin and their additive effects with tetracycline, however, on some of the strains only. The antileishmanial activity of compounds **2** and **4** was comparable with that of miltefosine. The most active compounds tested here were 3′-*O*-methyl substituted at the flavanone ring B. The activity of 3′-*O*-methyl substituted dihydroflavonol **3** was diminished probably due to the presence of 3-OH substitution; however, it is difficult to predict a structure–activity relationship for the compounds tested, because further assay showed that a small structural change of geranylated flavonoid at the ring B can affect the mechanism of antileishmanial effect. It is now clear that further studies to evaluate the mechanism of action and efficacy of these compounds both *in vitro* and *in vivo* are needed.

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