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Research Paper

Flavonoids in *Scutellaria immaculata* and *S. ramosissima* (Lamiaceae) and their biological activity

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Abstract

Objectives The aim of this study was to investigate the flavonoid composition of *Scutellaria immaculata* and *S. ramosissima* (Lamiaceae) and the in-vitro biological activity of their extracts and flavonoids.

Methods The flavonoid composition of *S. immaculata* (Si) and *S. ramosissima* (Sr) were analysed using LC-MS. Antimicrobial activity was studied *in vitro* against a range of bacteria and fungi using diffusion and microdilution methods. Anti-trypanosomal and cell proliferation inhibitory activity of the extracts and flavonoids was assessed using MTT. The antioxidant activity of the flavonoids and extracts were evaluated using DPPH* test.

Key findings LC-MS investigation of Si and Sr plants allowed the identification, for the first time, of an additional 9 and 16 flavonoids, respectively. The methanol, chloroform and water extracts from these plants and six flavonoids (scutellarin, chrysin, apigenin, apigenin-7-*O*-glucoside, cynaroside and pinocembrine) exhibited significant inhibition of cell growth against HeLa, HepG-2 and MCF-7 cells. The chloroform extract of Sr showed potent cytotoxic effects with IC50 (drug concentration which resulted in a 50% reduction in cell viability) values of 9.25 ± 1.07 μg/ml, 12.83 ± 1.49 μg/ml and 17.29 ± 1.27 μg/ml, respectively. The highest anti-trypanosomal effect against *T. b. brucei* was shown by the chloroform extract of Sr with an IC50 (drug concentration which resulted in a 50% inhibition of the biological activity) of 61 μg/ml. The pure flavonoids showed an IC50 range between 3 and 29 μM, with cynaroside as the most active compound with an IC50 value of 3.961 ± 0.133 μM. The chloroform extract of Sr has potent antimicrobial activity against *Streptococcus pyogenes* (minimum inhibitory concentration, MIC = 0.03 mg/ml). Pinocembrine exhibited a strong activity against the all bacteria except *Escherichia coli* and yeasts. Water extracts of Sr and Si exhibited potent antioxidant activity with IC50 values of 5.62 ± 0.51 μg/ml and 3.48 ± 0.02 μg/ml, respectively. Scutellarin exerted stronger antioxidant activity than other flavonoids.

Conclusions This is the first study reporting an in-vitro biological investigation for Si and Sr. Especially the chloroform extract of Sr showed potent anticancer and antimicrobial activity. Cynaroside had a highly selective and strong cytotoxicity against *T. b. brucei* while showing only mild effects against cancer cells.

Keywords antimicrobial; anti-trypanosomal; cytotoxicity; *Scutellaria immaculata*; *S. ramosissima*

Introduction

The genus *Scutellaria* (Lamiaceae) comprises over 360 species, many of which are medicinally important. Essential oils, flavonoids, phenylethanoid glycosides, iridoid glycosides, diterpenes, triterpenoids, alkaloids, phytosterols, polysaccharides and other secondary compounds have been isolated from *Scutellaria* species.[1] Some *Scutellaria* species are used to treat neurological disorders, cancer, inflammatory diseases and viral and bacterial infections. Extracts from *Scutellaria baicalensis* and *S. barbata* inhibited cancer cell proliferation and

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showed anti-tumour effects.[2] Previous studies demonstrated anti-trypanosomal effects of *S. baicalensis*.[3,4]

The pharmacological effects of *Scutellaria* species are attributed particularly to flavonoids, which are abundant in this genus. More than 60 flavonoids have been described in *S. baicalensis* and the most commonly studied flavonoids from this plant include baicalein, baicalin, wogonin and wogonoside. Flavonoids of *Scutellaria* have received scientific attention as modifiers of inflammatory processes, as well as for their antiviral, anti-retroviral, anti-tumour and antibacterial properties.[1]

These compounds show almost no or minor toxicity to normal epithelial and normal peripheral blood and myeloid cells. The anti-tumour functions of these flavones are largely due to their ability to scavenge oxygen radicals, attenuate NF-kappaB activity, suppress COX-2 gene expression, inhibit several genes important for regulation of the cell cycle, and to prevent viral infections.[5] Baicalein and baicalin were shown to protect several types of tissue against damage from reactive oxygen species (ROS)[6] and these flavonoids are reported to be largely responsible for the antimicrobial effects.[7,8] Baicalein has also been shown to inhibit HIV-1 reverse transcriptase.[9]

In Uzbekistan alone, 32 *Scutellaria* species have been found and some of them are considered as medicinal plants.[10] These plants are used in Uzbek traditional medicine to treat epilepsy, inflammation, allergies, chorea, nervous tension states and high blood pressure.[11] *S. immaculata* Nevski ex Juz. and *S. ramosissima* M. Pop. are perennial shrubs that grow in Northern Tien Shan, Pamirs and Altay mountains (Central Asia). Chemical analysis of these plants indicate the presence of flavonoids.[12–16] Although, there are widespread reports about the anti-tumour, antibacterial and antioxidant effects of some species of this genus, in-vitro biological studies have not yet been conducted on *S. immaculata* and *S. ramosissima*.

This study aimed to analyse the flavonoid composition of the methanol, chloroform and water extracts of the aerial parts (Srap), roots of *S. ramosissima* (Srr), and aerial parts of *S. immaculata* (Siap) using LC-MS. In addition in-vitro biological properties (cytotoxic, antioxidant, anti-trypanosomal and antimicrobial activity) of the extracts and flavonoids were evaluated in detail.

Materials and Methods

Plant material

The aerial parts and roots of *S. ramosissima* (Accession no. 20088052) and aerial parts of *S. immaculata* (Accession no. 20088045) employed in this investigation were collected from Tashkent and Namangan region of Uzbekistan, respectively, at the flowering stage during the summer of 2008. Plants were identified in the Department of Herbal Plants (Institute of the Chemistry of Plant Substances, Uzbekistan) by Dr O.A. Nigmatullaev and voucher specimens were deposited at this Department.

Chemicals

Media and supplements for cell cultures, doxorubicin (≥98%) and quercetin (≥98%) were obtained from Gibco (Invitrogen, Karlsruhe, Germany). Scutellarin and apigenin (≥95%) were purchased from Sigma Aldrich (Sternheim, Germany), chrysin (≥97%) and apigenin-7-*O*-glucoside were from Roth (Karlsruhe, Germany). Cynaroside and pinocembrine (≥95%) were obtained from the Institute of the Chemistry of Plant Substances, Tashkent, Uzbekistan.

Preparation of crude plant extracts

The plant material (root or aerial parts) was air-dried at room temperature before grinding it to a fine powder with a Waring blender. After grinding, 100 g of plant material was extracted with 500 ml solvent (methanol, chloroform or water). Extraction with each solvent was carried out for one day. The solvent was evaporated in a rotary vacuum evaporator at 40°C. Yields were calculated as %. The extracts were then kept in a refrigerator until further use.

HPLC analysis of flavonoids

The final concentration of all samples was 20 µg in 1 ml methanol. The HPLC system (Merck-Hitachi L-6200A) consisted of a *Rheodyne* injector (20 µl loop). Separation was carried out using a RP-C18$_e$ LichroCART 250–4, 5 µm column (Merck, Darmstadt, Germany). The mobile phase consisted of A: water (VWR, Fontenay-Sous-Bois, France) HPLC grade with 0.5% formic acid (Merk, Darmstadt, Germany), B: acetonitrile (J. T. Backer, Deventer, Holland). The gradient program was as follows: for methanol and chloroform fractions, from 0% to 50% B in 50 min then to 100% in 5 min; for water fraction, from 0% to 25% in 50 min then to 100% in 5 min.

Mass-spectrometry

A Quattro II system from VG with an ESI interface was used in a negative ion mode under the following conditions: drying and nebulizing gas, N_2; capillary temperature, 120°C; capillary voltage, 3.00 kV; lens voltage, 0.5 kV; cone voltage 30 V; full scan mode in mass range m/z 200–1000.

Cytotoxicity assay

Cell cultures

HeLa (cervical cancer), HepG-2 (hepatic cancer) and MCF-7 (breast cancer) cell lines were maintained in DMEM complete media (L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin) in addition to 10 mM non-essential amino acids. Bloodstream forms of *Trypanosoma brucei* TC221 cells (causative organism of the epidemic nagana) were grown in BALTZ medium[17] supplemented with 20% inactivated fetal bovine serum (FBS) and 0.001% β-mercaptoethanol. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. All experiments were performed with cells in the logarithmic growth phase.

Cytotoxicity and cell proliferation assay

The cytotoxicity of extracts and isolated compounds was determined in triplicate using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay.[18] The extracts and flavonoids were dissolved in dimethyl sulfoxide (DMSO) and further serially diluted with the medium in two-fold fashion into ten different concentrations so as to attain final concentrations ranging from 0.977

to 500 μg/ml for extracts and from 0.977 to 500 μM for individual substances, in 96-well plates. A 100 μl sample was dispensed into each well. The concentration of the solvent, DMSO, did not exceed 0.05% in the medium that contained the highest concentration of extract or compound tested. Wells containing the solvent and wells without the solvent were included in the experiment. Cells (2×10^4 cells/well of exponentially growing cells of each individual HeLa, HepG-2, MCF-7, *T. b. brucei*, TC221 cells) were seeded in a 96-well plate (Greiner Labortechnik), the cells were cultivated for 24 h and then incubated with various concentrations of the serially diluted tested samples at 37°C for 24 h and then with 0.5 mg/ml MTT for 4 h. The formed formazan crystals were dissolved in 100 μl DMSO. The absorbance was detected at 570 nm with a Tecan Safire II Reader. The cell viability rate (%) of three independent experiments was calculated by the following formula:

$$\text{Cell viability rate (\%)} = (\text{OD of treated cells}/\text{OD of control cells}) \times 100\% \quad (1)$$

The *T. b. brucei* TC221 viability results were additionally confirmed by counting cells under a light microscope. Diminazene, suramin and doxorubicin were used as positive controls.

Antimicrobial activity

Test microorganisms

The antimicrobial activity was evaluated using standard strains: the Gram-positive bacteria *Streptococcus pyogenes* ATCC 12344 and methicillin-resistant *Staphylococcus aureus* NTCC 10442; the Gram-negative bacteria *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853; and the fungi *Candida albicans* ATCC 90028 and *Candida glabrata* ATCC MYA 2950. All microorganism cultures were supplied by Medical Microbiology Laboratory, Hygiene Institute, University of Heidelberg, Germany.

Culture media

Columbia medium with 5% sheep blood (BD) was used for bacteria sub-culturing and minimum bactericidal concentration (MBC) determination. Mueller-Hinton Broth (MHB) (Fluka) was used for bacterial inocula dilution and minimum inhibitory concentration (MIC) determination. All bacterial cultures were incubated at 37°C for 24 h. CHROM agar Candida (BD) was used for sub-culturing fungi and MBC determination. Sabouraud dextrose broth (SDB) (Oxid) was used for fungal inocula dilution and MIC determination. All fungal cultures were incubated at 25°C for 48 h.

Inoculum preparation

One or two colonies from an 18–24 h agar plate were suspended in saline to a turbidity matching 0.5 McFarland $\approx 1 \times 10^8$ colony-forming units (CFU)/ml, then 100 μl of this suspension was diluted to 1 : 100 with 900 μl broth to obtain 1×10^6 CFU/ml.[19]

Diffusion method

Columbia medium with 5% sheep blood and CHROM agar Candida were inoculated with 1×10^6 CFU/ml of bacterial and fungal suspensions, respectively. Wells with diameter of 6 mm were cut off and delivered with 40 μl of each extract (16 mg/ml) and of each pure substance (1 mM). Ampicillin (1 mg/ml), vancomycin (1 mg/ml), nystatin (1 mg/ml) and DMSO were used as controls. The diameters of the growth inhibition zones were measured in triplicate after incubation at 37°C for 24 h (bacteria) or 48 h (yeast).

Determination of minimum inhibitory concentration and minimum microbicidal concentration

Micro-dilution method was used to determine MIC as described by CLSI.[19] Plant extracts and compounds were first dissolved in DMSO 5% to 8 mg/ml and then were diluted two fold with MHB (bacteria) and SDB (fungi) in 96-well plates to obtain a range of concentrations (4–0.007 mg/ml). The bacterial and fungal suspensions of 1×10^6 CFU/ml were then added and the plates were incubated at 37°C for 24 h (bacteria) or at 25°C for 48 h (fungi). The concentration in the first well that showed no visible turbidity matching with a negative control was defined as the MIC. Three microlitres from each clear well (with no visible turbidity) were inoculated in appropriate agar media and incubated under the appropriate conditions. The minimum microbicidal concentration (MMC) was determined as the concentration that showed no growth on agar after incubation. Each test was performed in triplicate.

Antioxidant activity

The antioxidant and radical scavenging activity of flavonoids and extracts was evaluated according to a previous described method using diphenyl picryl hydrazyl (DPPH*).[20] Equal volumes of sample solutions containing 0.02–10 mg/ml of the tested samples and 0.2 mM methanolic solution of DPPH* were pipetted into 96-well plates. The absorbance was measured against a blank at 517 nm using a Tecan Safire II Reader after incubation in the dark for 30 min at room temperature compared with DPPH* control after background subtraction. Quercetin was used as a positive control. The percent inhibition was calculated from three different experiments using the following equation:

$$\text{RSA (\%)} = [(\text{Abs}_{517\text{control}} - \text{Abs}_{517\text{sample}})/\text{Abs}_{517\text{control}}] \times 100 \quad (2)$$

where RSA = radical scavenging activity; Abs_{517} = absorption at 517 nm

Statistical analysis

All experiments were carried out three times unless mentioned in the procedure. Continuous variables were presented as mean ± SD. The IC50 was determined as the drug concentration that resulted in a 50% reduction in cell viability or inhibition of the biological activity. IC50 values were calculated using a four parameter logistic curve (SigmaPlot 11.0) and all the data were statistically evaluated using Student's *t*-test or the Kruskal–Wallis test (GraphPad Prism 5.01; GraphPad Software, Inc., San Diego, USA) followed by Dunn's post-hoc multiple comparison test when the significance value was <0.05 using the same significance level. The criterion for statistical significance was generally taken as $P < 0.05$.

Results

Identification of flavonoids

The flavonoids from *S. immaculata* and *S. ramosissima* (Figure 1) were identified by comparison of their LC-MS spectra with published data or were confirmed by comparison with authentic samples.[21,22] (Figures 2–4). The LC-MS investigation of *S. ramosissima* allowed the identification of the following additional flavonoids: chrysin-6-arabinosyl-8-*C*-glucoside (**4**), isorhamnetin-7-*O*-rhamnosyl-glucoside (**5**), rhamnetin-7-*O*-rhamnosyl-glucoside (**6**), scutellarin (**8**), baicalin (**9**), 5,7,2′,5′-tetrahydroxy-8,6′-dimethoxyflavone (**10**), oroxylin A-7-*O*-glucoside (**11**), 5,6,7-trihydroxy-flavanone (dihydroxybaicalein)-7-*O*-glucuronide (**12**), norwogonin-7-*O*-glucuronide (**13**), chrysin-7-*O*-glucuronide (**14**), oroxylin A-7-*O*-glucuronide (**15**), wogonin-7-*O*-glucuronide (**16**), norwogonin (**21**), 5,7,3-trihydroxy-4′-methoxyflavone (**22**), baicalein (**23**), 5,7,4′-trihydroxy-8-methoxyflavone (**24**), wogonin (**25**), chrysin (**26**) and 5,2′-dihydroxy-6,7,8-trimethoxyflavone (**27**) (Tables 1 and 2 and Figure 1). Flavonoids **1**–**7**, **9**–**14** and **16**–**27** from aerial parts and **3**–**8**, **10**–**16** and **18**–**27** from roots were identified in *S. ramosissima* for the first time (Figures 2–4). Flavonoids **14**, **21** and **25** had been isolated before from the aerial and roots from this plant.[12–14,16]

In this study we have recorded flavonoids **1**, **8**, **9**, **11**–**14**, **16**, **18**–**20**, **21**–**23**, **25** and **26** from aerial parts of *S. immaculata* for the first time (Tables 1 and 2, and Figures 2–4). The occurrence of flavonoids **14**, **25**, and **26** in *S. immaculata* is in agreement with the previously reported flavonoid profile.[15,16]

Cytotoxic and anti-trypanosomal activity

The anti-proliferative activity of nine extracts from aerial parts and roots of *S. ramosissima* and from aerial parts of *S. immaculata*, including six isolated flavonoids scutellarin (**8**), chrysin (**26**), apigenin (**28**), apigenin-7-*O*-glucoside (**29**),

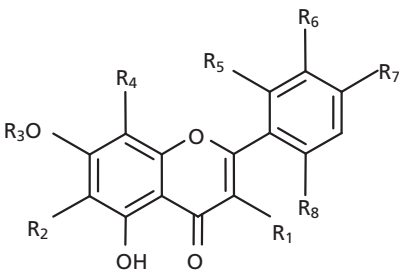
Table 1 Identification of flavonoids in *Scutellaria* species by LC-MS

Peak	$[M-H]^-_{m/z}$	Compound	Reference
1	345	Unknown	–
2	431	Unknown	–
3	341	Unknown	–
4	547	Chrysin-6-arabinosyl-8-C-glucoside	17
5	623	Isorhamnetin-7-*O*-rha-glu	16
6	623	Rhamnetin-7-*O*-rha-glu	16
7	637	Unknown	–
8	461	Scutellarin	–
9	445	Baicalin	17
10	345	5,7,2′,5′-Tetrahydroxy-8,6′-dimethoxy flavone	17
11	445	Oroxylin A-7-*O*-glucoside	17
12	447	5,6,7-Trihydroxy flavanone(dihydroxybaicalein)-7-*O*-glucuronide	17
13	445	Norwogonin-7-*O*-glucuronide	17
14 + **15**	429 + 459	Chrysin-7-*O*-glucuronide + oroxylin A-7-*O*-glucuronide	17
16	459	Wogonin-7-*O*-glucuronide	17
17	431	Unknown	–
18	329	Unknown	–
19	327	Unknown	–
20	359	Unknown	–
21 + **22**	269 + 299	Norwogonin + 5,7,3-trihydroxy-4′-methoxyflavone	16
23 + **24**	269 + 299	Baicalein +5,7,4′-trihydroxy-8-methoxyflavone	16
25	283	Wogonin	17
26	253	5,7-Dihydroxyflavone (chrysin)	17
27	343	5,2′-Dihydroxy-6,7,8-trimethoxyflavone	17

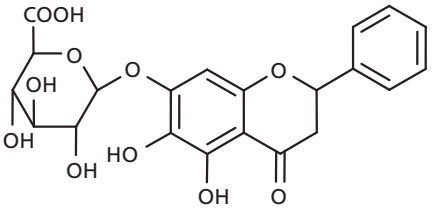
Table 2 Flavonoids in *S. immaculata* and *S. ramosissima*

Plant material	Extracts (yield, %)	Flavonoids
S. ramosissima (aerial parts)	$CHCl_3$ (3.5%)	**10**, **11**, **14**, **18**–**27**
	MeOH (7.2%)	**1**–**4**, **6**, **9**, **11**–**14**, **16**, **17**, **19**–**22**, **24**–**27**
	H_2O (4.5%)	**3**–**7**, **11**–**14**, **16**, **22**
S. ramosissima (roots)	$CHCl_3$ (2.3%)	**10**, **11**, **14**, **18**–**27**
	MeOH (11.8%)	**3**–**6**, **8**–**13**, **15**–**27**
	H_2O (5.3%)	**3**–**7**, **11**–**14**, **16**, **18**, **20**, **22**, **23**, **26**, **27**
S. immaculata (aerial parts)	$CHCl_3$ (2.1%)	**8**, **14**, **18**–**20**, **25**, **26**
	MeOH (8.1%)	**1**, **8**, **9**, **11**–**14**, **16**, **18**–**23**, **26**
	H_2O (3.9%)	**8**, **11**, **13**, **14**

Flavonoid numbers as in Table 1. %, Yield of the each extract was calculated on the basis of air-dried weight of the plant material (aerial part or roots).



4-6, 8-11, 13-16, 21-30



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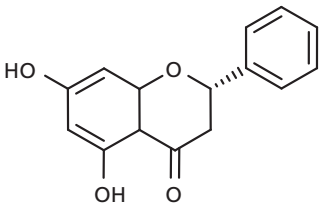

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	Substance	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
4	Chrysin-6-arabinosyl-8-C-glucoside	H	Ara	H	Glu	H	H	H	H
5	Isorhamnetin-7-O-rha-glu	OH	H	Rha-Glu	H	H	OCH_3	OH	H
6	Rhamnetin-7-O-rha-glu	Rha-Glu	H	OCH_3	H	H	OH	OH	H
8	Scutellarin	H	OH	Glu acid	H	H	H	OH	H
9	Baicalin	H	OH	Glu acid	H	H	H	H	H
10	5,7,2`,5`-Tetrahydroxy-8,6`-dimethoxy flavones	H	H	H	OCH_3	OCH_3	OH	H	OH
11	Oroxylin A-7-O-glucoside	H	OCH_3	Glu	H	H	H	H	H
13	Norwogonin-7-O-glucuronide	H	H	Glu acid	OH	H	H	H	H
14	Chrysin-7-O-glucuronide	H	H	Glu acid	H	H	H	H	H
15	Oroxylin A-7-O-glucuronide	H	OCH_3	Glu acid	H	H	H	H	H
16	Wogonin-7-O-glucuronide	H	H	Glu acid	OCH_3	H	H	H	H
21	Norwogonin	H	H	H	OH	H	H	H	H
22	5,7,3-Trihydroxy-4`-methoxyflavone	OH	H	H	H	H	H	OCH_3	H
23	Baicalein	H	OH	H	H	H	H	H	H
24	5,7,4`-Trihydroxy-8-methoxyflavone	H	H	H	OCH_3	H	H	OH	H
25	Wogonin	H	H	H	OCH_3	H	H	H	H
26	Chrysin	H	H	H	H	H	H	H	H
27	5,2`-Dihydroxy-6,7,8-trimethoxyflavone	H	OCH_3	OCH_3	OCH_3	OH	H	H	H
28	Apigenin	H	H	H	H	H	H	OH	H
29	Apigenin-7-*O*-glucoside	H	H	Glu	H	H	H	OH	H
30	Cynaroside	H	H	Glu	H	H	OH	OH	H

Figure 1 Chemical structures of flavonoids studied.

cynaroside (**30**) and pinocembrine (**31**), was determined in HeLa, HepG-2, MCF-7 cells and against *T. b. brucei*. The IC50 values are presented in Table 3.

SrapC, SrrC and SiapC substantially inhibited the proliferation of cancer cells. The IC50 values of SrapC in all three human cancer cell lines were in the range 9–17 μg/ml, those of SrrC were between 9 and 30 μg/ml and those of SiapC were between 13 and 31 μg/ml, with HepG-2 as the most sensitive and MCF-7 as the least sensitive cell line. The IC50 values against *T. b. brucei* were in the range 0.6–0.8 μg/ml. This results in a selectivity index for *T. b. brucei* in relation to the cancer cell lines in the range 13–50 (15–50 for SrapC, 13–24 for SrrC and 17–39 for SiapC). The highest selectivity index was obtained for *T. b. brucei* and MCF-7 cells and the lowest index was between *T. b. brucei* and HepG-2.

Chrysin and apigenin showed the strongest cytotoxicity of all tested flavonoids in all three human cancer cell lines and in *T. b. brucei*, while cynaroside was highly selective for *T. b. brucei* with only minor toxicity in all three human cancer cell lines. The IC50 values of chrysin in all three human cancer cell lines range between 13 and 22 μM and of apigenin between 17 and 64 μM, while the IC50 value of chrysin in *T. b. brucei* was 11 μM and of apigenin 8 μM. The resulting selectivity index for chrysin ranges between 1 and 2 and for apigenin between 2 and 7. Cynaroside was remarkably different from all tested flavonoids with IC50 values between 149 and 184 μM in the three cancer cell lines and an IC50 value of 4.9 μM in *T. b. brucei*. The selectivity index thus ranges between 30 and 37.

Antimicrobial activity

A total of six microbial strains (two Gram-positive, two Gram-negative bacteria, two fungi) were used in this investigation. Methanol, chloroform and water extracts of Siap, Srap, Srr and flavonoids **8**, **26** and **28–31** were tested at various concentrations, ranging from 4 to 0.007 mg/ml. MIC and MMC values are reported in Table 4.

All chloroform extracts showed strong growth inhibition against *Streptococcus pyogenes.* The SiapC exhibited moderate antimicrobial activity (MIC = 1 mg/ml) compared with SrapC and SrrC, which exhibited stronger antimicrobial activity (MIC = 0.06 and 0.03 mg/ml, respectively) against

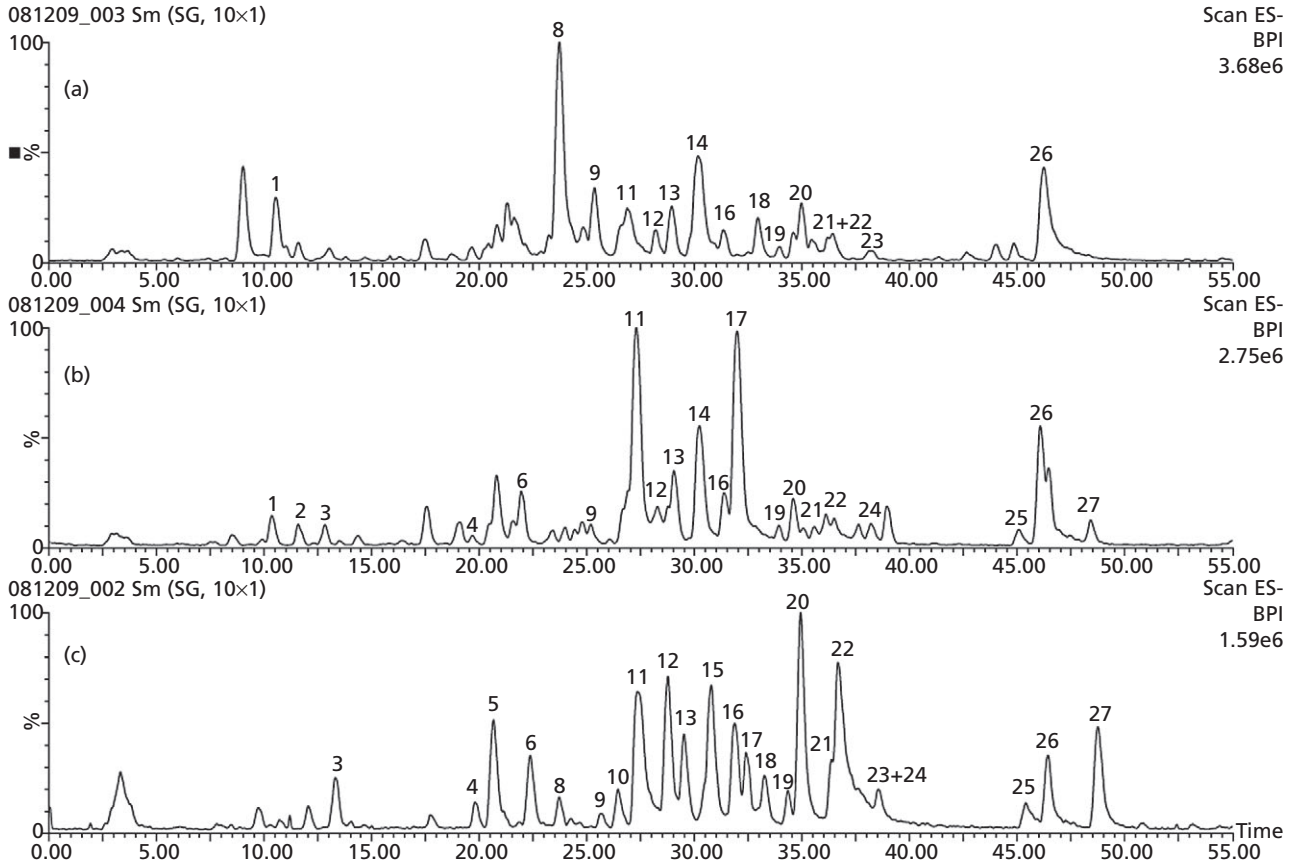

Figure 2 LC-MS (–) of methanol extracts. (a) *S. immaculata* aerial parts. (b) *S. ramosissima* aerial parts. (c) *S. ramosissima* roots.

Table 3 Anti-trypanosomal and anti-proliferative activity of *S. immaculata* and *S. ramosissima* extracts and isolated flavonoids

Sample	IC50 (μg/ml)			
	T. b. brucei	HeLa	HepG-2	MCF-7
S. immaculata (aerial parts)				
MeOH	1.785 ± 1.373	62.898 ± 2.860	42.81 ± 3.88	62.53 ± 0.47
$CHCl_3$	0.879 ± 0.329	20.552 ± 1.597	13.86 ± 1.15	31.52 ± 1.32
H_2O	3.645 ± 0.990	32.086 ± 0.829	32.06 ± 2.28	44.05 ± 3.25
S. ramosissima (aerial parts)				
MeOH	3.857 ± 1.574	74.571 ± 2.512	62.91 ± 6.27	69.73 ± 5.93
$CHCl_3$	0.611 ± 0.130	23.181 ± 1.543	9.04 ± 1.04	30.41 ± 0.37
H_2O	7.950 ± 2.656	86.365 ± 4.104	66.57 ± 4.70	93.19 ± 5.10
S. ramosissima (roots)				
MeOH	1.822 ± 1.094	66.573 ± 6.232	52.59 ± 4.20	53.01 ± 3.88
$CHCl_3$	0.723 ± 0.127	9.219 ± 0.934	12.83 ± 1.49	17.29 ± 1.27
H_2O	1.698 ± 0.689	57.197 ± 3.613	40.29 ± 3.44	54.30 ± 0.22
Diminazene (μg/ml) (positive control)	0.084 ± 0.012	170.663 ± 3.211	–	–
Doxorubicin (μg/ml) (positive control)	–	1.07 ± 0.11	0.39 ± 0.04	0.28 ± 0.02
Suramin (μg/ml) (positive control)	4.724 ± 0.129	1317.177 ± 9.411	–	–
	IC50 (μM)			
Apigenin (**28**)	8.670 ± 0.091	33.306 ± 4.323	64.56 ± 5.72	17.25 ± 1.33
Apigenin-7-*O*-glucoside (**29**)	29.692 ± 0.575	137.832 ± 4.981	291.61 ± 23.46	151.64 ± 14.87
Chrysin (**26**)	11.431 ± 0.399	22.808 ± 3.793	13.98 ± 1.09	22.73 ± 2.48
Cynaroside (**30**)	4.939 ± 0.106	148.990 ± 12.773	151.64 ± 14.19	184.13 ± 17.37
Pinocembrine (**31**)	36.999 ± 1.017	109.825 ± 10.165	150.97 ± 15.19	171.63 ± 11.78
Scutellarin (**8**)	27.864 ± 1.123	127.826 ± 7.221	94.60 ± 5.16	31.84 ± 3.35
Doxorubicin (μM) (positive control)	–	1.84 ± 0.19	0.67 ± 0.07	0.48 ± 0.04

IC50, values (μg/ml) of the anti-trypanosomal and cytotoxicity effects of flavonoids and extracts from *S. immaculata* and *S. ramosissima*. The data shown are means ± SD obtained from three independent experiments.

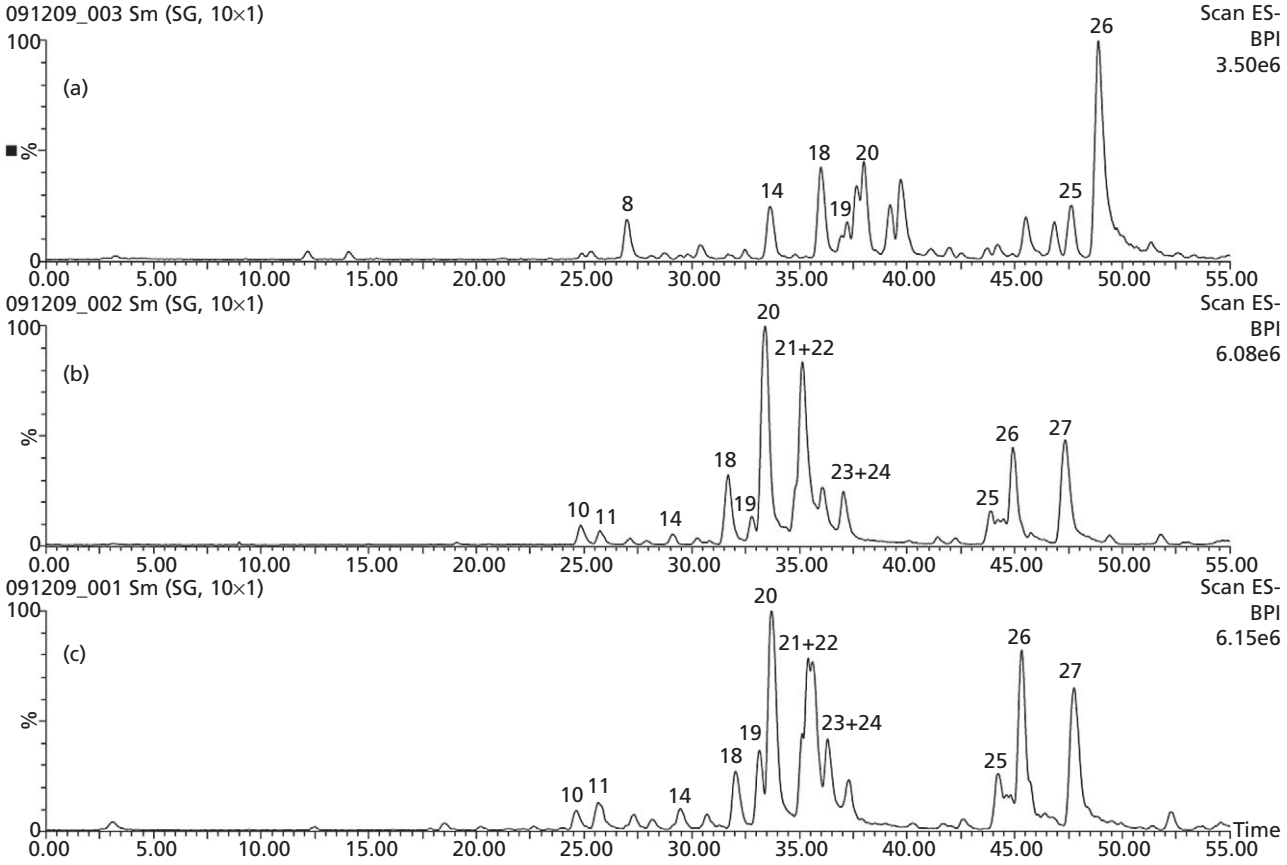

Figure 3 LC-MS (–) of chloroform extracts. (a) *S. immaculata* aerial parts. (b) *S. ramosissima* roots. (c) *S. ramosissima* aerial parts.

S. pyogenes. SiapM, SrapM and SrrM exhibited significant antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa* and *S. pyogenes.* Most of the plant extracts showed weak inhibition (MIC >3 mg/ml) against *P. aeruginosa*; only SrapC (MIC = 1 mg/ml) was found to have moderate activity. Data in Table 4 indicate a weak activity against *Candida albicans* only for SrrC and SrrM (MIC >4 mg/ml), while the other extracts were inactive against this strain. *Candida glabrata* was resistant to the SiapM, SrrM and SrapW, while the other extracts showed a weak inhibition (MIC >2 mg/ml) against this yeast.

The antimicrobial activity of flavonoids (inhibition zones, MIC and MMC values) is shown in Table 4. Apigenin-7-*O*-glucoside (**29**) exhibited a strong activity against all the bacteria and yeasts (MIC = 0.25–0.5 mM). Cynaroside (**30**) was active against *S. aureus*, *E. coli, P. aeruginosa* and *S. pyogenes* with MIC = 0.5 mM and had no activity on fungal strains. Pinocembrine (**31**) showed potent antimicrobial activity against *S. pyogenes, S aureus*, *C. albicans* and *C. glabrata* (MIC = 0.25 mM). Apigenin (**28**), was inactive against *S. aureus*, *P. aeruginosa* and *C. albicans* and proved to be the most active substance against *S. pyogenes, E. coli* and *C. glabrata* (MIC = 0.25–0.5 mM). Scutellarin (**8**) showed strong growth inhibition against *S. pyogenes, E. coli, C. albicans* and *C. glabrata.* Only chrysin (**26**) was inactive against any of the bacterial pathogens at the concentrations tested.

Antioxidant activity

The free radical scavenging activity was determined using DPPH* assay. The DPPH* scavenging capacity of the extracts and flavonoids were compared with the known antioxidative substance quercetin. The DPPH* radical-scavenging activity of the extracts and flavonoids studied are shown in Table 5. All plants extracts possessed significant DPPH* radical scavenging activity. The most active plant extract was SiapW (IC50 = 3.48 ± 0.02 μg/ml) compared with the most active single compound scutellarin (4.77 ± 0.53 μg/ml).

Discussion

Scutellaria species are characterized by their richness in flavonoids. Malikov and Yuldashev reported 208 phenolic compounds from *Scutellaria baicalensis*, which has the highest number of known phenolic compounds (>60) and represents the best studied species in the genus.[15]

S. immaculata has been studied previously and 17 flavonoids (chrysin-7-*O*-glucuronide, scutellarein-7-*O*-glucoside, apigenin-7-*O*-glucoside, baicalein-7-*O*-glucoside, norwogonin-7-*O*-glucoside, oroxyloside, wogonoside, immaculoside, 5,2′-dihydroxy-6,7,6′-trimethoxyflavanone, 5,2′-dihydroxy-6,7,8,6′-tetramethoxyflavanone, chrysin, wogonin, apigenin, isoscutellarein, scutellarein, cosmosiin (apigenin-7-*O*-β-D-glucopyranoside), and wogonin-

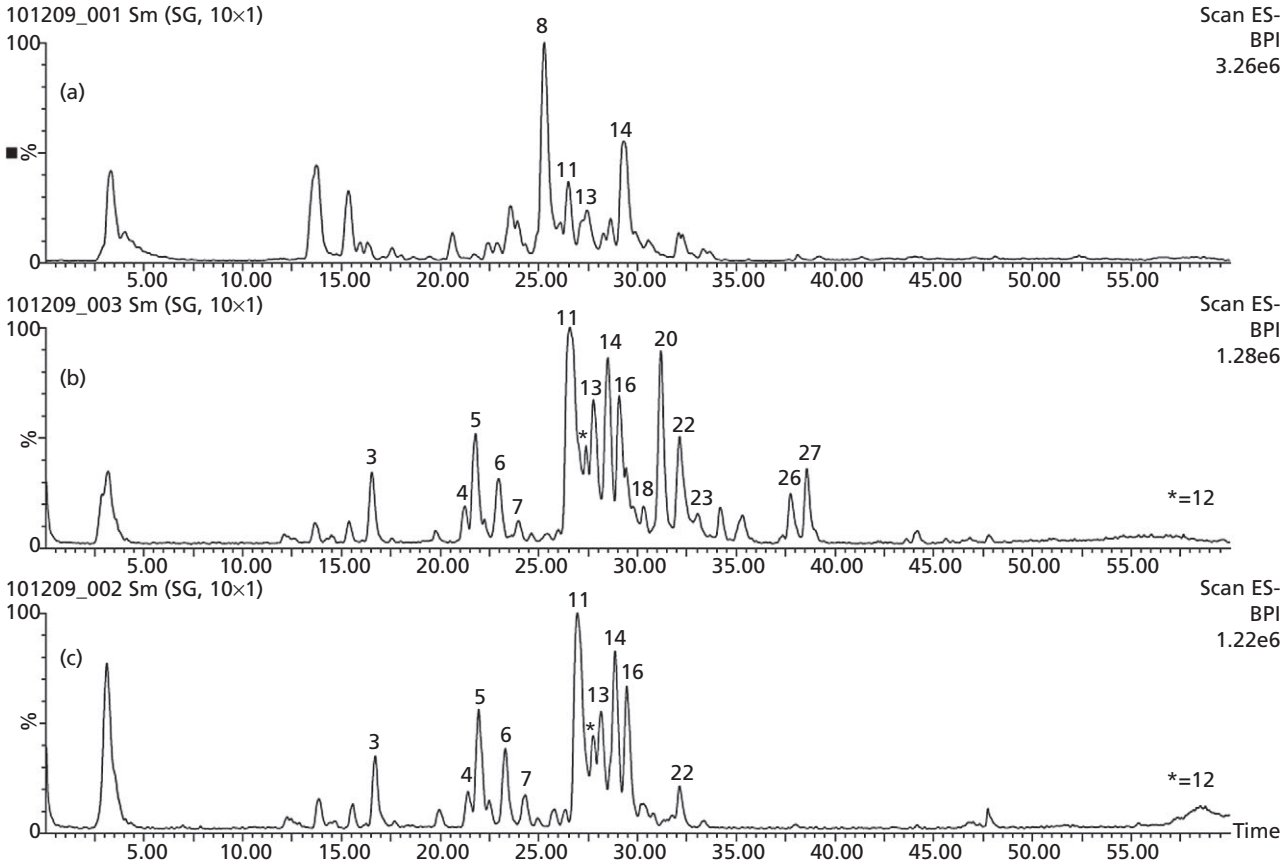

Figure 4 LC-MS (–) of water extracts. (a) *S. immaculata* aerial parts. (b) *S. ramosissima* roots. (c) *S. ramosissima* aerial parts.

7-O-β-D-glucopyranoside) were identified;[15,16] and from *S. ramosissima*, chrysin 7-*O*-β-D-glucuronide, 2(S)-2′,5,7-trihydroxyflavanone 7-*O*-(Me β-D-glucopyranosiduronate), 2(S)-2′,5,7-trihydroxyflavanone 7-*O*-(Et β-D-glucopyranosiduronate), 5,2′-dihydroxy-7-*O*-β-D-glucopyranosylflavone, rivularin, 5,2′-dihydroxy-7-*O*-β-D-glucopyranosylflavanone, oroxylin A, wogonin, norwogonin, 5,2′,6′-trihydroxy-6,7,8-trimethoxyflavone, and 5,6-dihydroxy-7,8-dimethoxyflavone.[12,13,23] However, flavonoids have not been determined fully in *S. immaculata* and *S. ramosissima*. In this investigation evidence for the presence of an additional 19 and 12 flavonoids from *S. immaculata* and *S. ramosissima*, respectively, is provided (Tables 1 and 2). Our data confirm that *Scutellaria* is characterized by a substantial accumulation and a broad structural diversity of flavonoids. Their biological properties vary considerably with only minor modifications in their structure. The number and specific positions of phenolic hydroxyl groups and the nature of the substitutions determine whether flavonoids function as strong antioxidative,[24–26] anti-inflammatory, anti-proliferative or enzyme-modulating agents.[27–30] The phenolic hydroxyl groups of flavonoids can dissociate to negatively charged phenolate ions under physiological conditions. Therefore, flavonoids can interact with several proteins (including transporters, enzymes and transcription factors) by binding to them via hydrogen bridges and ion bonding. As a result the conformation of proteins are disturbed and in consequence their biological activity is altered.[31] These physicochemical properties can explain the wide range of activities of polyphenols. Flavonoids are well known for their wide range of biological activity. Recent studies regarding the cytotoxic activity of the flavonoids of *Scutellaria* indicate that some flavonoids, like baicalein,[32,33] wogonin[34] and luteolin,[35] possess substantial anti-cancer activity. For example, baicalein showed substantial toxicity to P-glycoprotein or MRP1-expressing multidrug-resistant cells.[36] The anti-tumour effects of these flavonoids may be due to their ability to inhibit several genes important for the regulation of the cancer cell cycle, and to prevent viral infections.[5]

The cytotoxicity of chloroform extracts was superior to that of the methanol and water extracts. Especially, the SrrC showed a high toxicity level in all cell lines (Table 3). Chrysin (**26**) and norwogonin (**21**) might be responsible for the toxicity since they are the main compounds of the chloroform extracts while the methanol and water extracts mainly contain scutellarin (**8**) and oroxylin A-7-*O*-glucoside (**11**). Chrysin (**26**) was the most cytotoxic single substance tested with a cytotoxicity ranging between 11 μM in *T. b. brucei* and 22 μM in HeLa cells, which makes it 12 times less toxic than the positive control doxorubicin (1.84 μM).

Table 4 Minimum inhibitory concentrations (MIC) and minimum microbicidal concentrations (MMC) of the flavonoids, *S. immaculata* and *S. ramosissima* plant extracts against different pathogens using the broth microdilution method

Sample	Extract	G$^+$ *Staphylococcus aureus* MRSA ATCC 10442			G$^+$ *Streptococcus pyogenes* ATCC 12344			G$^-$ *Escherichia coli* ATCC 25922			G$^-$ *Pseudomonas aeruginosa* ATCC 27853			Yeast *Candida albicans* ATCC 90028			Yeast *Candida glabrata* ATCC MYA 2950		
		I.z. (mm)	MIC mg/ml	MMC mg/ml	I.z. (mm)	MIC mg/ml	MMC mg/ml	I.z. (mm)	MIC mg/ml	MMC mg/ml	I.z. (mm)	MIC mg/ml	MMC mg/ml	I.z. (mm)	MIC mg/ml	MMC mg/ml	I.z. (mm)	MIC mg/ml	MMC mg/ml
S. immaculate (aerial parts)	MeOH	5 ± 0.5	2	4	5 ± 1	2	4	4 ± 0.8	2	4	4 ± 0.6	3	4	NA	NA	NA	NA	NA	NA
	$CHCl_3$	6 ± 1	1	4	7 ± 0.5	1	2	0	4	>4	5 ± 0.2	3	4	NA	NA	NA	0	4	>4
	H_2O	5 ± 1	2	4	5 ± 0.2	2	5	4 ± 0.5	2	4	4 ± 1	3	4	NA	NA	NA	5 ± 1	2	4
S. ramosissima (aerial parts)	MeOH	4 ± 0.8	2	4	5 ± 1.5	1	3	4 ± 0.6	2	4	4 ± 0.5	4	5	NA	NA	NA	4 ± 0.5	>4	>4
	$CHCl_3$	8 ± 1	0.5	4	7 ± 2	0.06	0.5	NA	NA	NA	6 ± 0.8	1	4	NA	NA	NA	5 ± 0.3	2	4
	H_2O	4 ± 0.6	2	4	5 ± 0.2	4	4	0	4	>4	4 ± 0.8	4	>4	NA	NA	NA	NA	NA	NA
S. ramosissima (roots)	MeOH	5	1	5	5 ± 0.2	1	4	4 ± 0.5	2	>4	4 ± 0.0	3	4	5	>4	>4	NA	NA	NA
	$CHCl_3$	8 ± 1	0.5	4	8 ± 1	0.03	0.5	NA	NA	NA	5 ± 0.5	1	4	5 ± 0.2	>4	>4	0	4	>4
	H_2O	5 ± 1	1	2	4 ± 1	2	5	0	4	>4	4 ± 0.2	3	4	NA	NA	NA	0	4	>4
Positive controls		I.z. (mm)	MIC µg/ml	MMC µg/ml	I.z. (mm)	MIC µg/ml	MMC µg/ml	I.z. (mm)	MIC µg/ml	MMC µg/ml	I.z. (mm)	MIC µg/ml	MMC µg/ml	I.z. (mm)	MIC µg/ml	MMC µg/ml	I.z. (mm)	MIC µg/ml	MMC µg/ml
Ampicillin		0	25	>25	25 ± 1	0.05	0.1	5 ± 1	12.5	25	NA	NA	NA	NT	NT	NT	NT	NT	NT
Vancomycin		10 ± 0.2	0.8	12.5	15 ± 1	0.1	0.4	NA	NA	NA	NA	NA	NA	NT	NT	NT	NT	NT	NT
Nystatin		NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	10 ± 1.2	0.2	0.4	12 ± 1	0.2	0.2
Substance		G$^+$ *Staphylococcus aureus* MRSA ATCC 10442			G$^+$ *Streptococcus pyogenes* ATCC 12344			G$^-$ *Escherichia coli* ATCC 25922			G$^-$ *Pseudomonas aeruginosa* ATCC 27853			Yeast *Candida albicans* ATCC 90028			Yeast *Candida glabrata* ATCC MYA 2950		
		I.z. (mm)	MIC mM	MMC mM	I.z. (mm)	MIC mM	MMC mM	I.z. (mm)	MIC mM	MMC mM	I.z. (mm)	MIC mM	MMC mM	I.z. (mm)	MIC mM	MMC mM	I.z. (mm)	MIC mM	MMC mM
Apigenine (**28**)		NA	NA	NA	4 ± 0.5	0.5	>0.5	0	0.5	>0.5	NA	NA	NA	NA	NA	NA	5 ± 1	0.25	0.5
Apigenine-7-*O*-glucoside (**29**)		0	0.5	>0.5	5	0.5	>0.5	0	0.5	0.5	0	0.5	>0.5	5 ± 1	>0.5	>0.5	5 ± 1	0.25	0.5
Chrysin (**26**)		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cynaroside (**30**)		0	0.5	>0.5	0	0.5	>0.5	0	0.5	>0.5	0	0.5	>0.5	NA	NA	NA	NA	NA	NA
Pinocembrine (**31**)		0	0.25	1	0	0.25	1	NA	NA	NA	0	0.5	>0.5	4 ± 1	0.25	0.25	5	0.25	0.25
Scutellarin (**8**)		NA	NA	NA	4 ± 0.3	0.5	>0.5	0	0.5	>0.5	NA	NA	NA	6 ± 0.5	>0.5	>0.5	5 ± 0.6	0.25	0.5

I.Z., inhibition zone; NA: not active; NT: not tested.

Table 5 Antioxidant activity of pure isolated of the flavonoids, *S. immaculata* and *S. ramosissima* plant extracts and isolated flavonoids using the DPPH* radical scavenging assay

Sample	IC50 (μg/ml)
S. immaculata (aerial parts)	
MeOH	6.41 ± 0.62
$CHCl_3$	30.09 ± 3.21
H_2O	3.48 ± 0.02
S. ramosissima (aerial parts)	
MeOH	9.62 ± 0.98
$CHCl_3$	13.86 ± 1.43
H_2O	5.62 ± 0.51
S. ramosissima (roots)	
MeOH	10.77 ± 1.12
$CHCl_3$	12.88 ± 1.50
H_2O	5.81 ± 0.53
Apigenin (**28**)	206.17 ± 18.12
Apigenin-7-*O*-glucoside (**29**)	286.54 ± 25.96
Chrysin (**26**)	308.27 ± 28.34
Cynaroside (**30**)	13.90 ± 1.46
Pinocembrine (**31**)	413.01 ± 35.21
Scutellarin (**8**)	4.77 ± 0.53
Quercetin (positive control)	3.37 ± 0.77

Data are shown as means ± S.D. of IC50 values (μg/ml).

As was previously shown, flavonoids are potential lead structures for the discovery of new trypanocidal drugs.[37] The activity of flavonoids is closely linked to their structure and the number of phenolic OH groups.[38] The flavonoids with a high number of phenolic OH groups were especially active in all human cancer cell lines and in *T. b. brucei* (Table 3). Chrysin (**26**) and apigenin (**28**) were most potent in both human cancer cell lines and in *T. b. brucei*, while cynaroside (**30**) was highly selective with mild cytotoxicity in human cancer cell lines (IC50 values 149–184 μM) but showing strong cytotoxicity in *T. b. brucei* (4.9 μM). The selectivity index between 30 and 37 seems promising for a potential anti-trypanosomal use of cynaroside (**30**). Suramin, still used as a standard drug against trypanosomiasis with a selectivity index of 280 and a toxicity of 4.7 μg/ml in *T. b. brucei*, is known to cause severe side effects. Thus, new lead structures for the treatment of trypanosomiasis are urgently required.

Comparison of the different flavonoid molecules shows that the R6 hydroxyl group of cynaroside (**30**) is missing in all inactive flavonoids. We suggest that this R6 hydroxyl group forms a crucial part in the activity against *T. b. brucei* and explains the high selectivity between cancer cells and *T. b. brucei* while all other flavonoids are less selective. It is probable that the activity of the extracts is caused not by only a single flavonoid but rather several ones working together synergistically. Further research should be conducted to confirm the structural dependency of the effect of flavonoids on *T. b. brucei.*

Compared with antibiotics (positive control), the broth micro-dilution method showed that all plant extracts have antimicrobial properties with an inhibition zone (I.z.), ranging from 4 to 8 mm. The MIC values of the pure compounds are in the same range as the tested antibiotics, thus making them promising candidates for lead structures. SrrC was the most active against *Staphylococcus aureus* and *S. pyogenes*, followed by SrapC and SiapC (Table 4).

The pure flavonoids showed a wide spectrum of activity against both yeast and bacteria (Table 4). The strong activity of apigenin (**28**), apigenin-7-*O*-glucoside (**29**), pinocembrine (**31**) and scutellarin (**8**) against yeast is promising. All of them have the R7 hydroxyl group, which might play an important role in their activity. Apigenin-7-*O*-glucoside (**29**) is highly active against all strains of bacteria and yeast. Its higher activity compared with its aglycon results from the higher bioavailability due to the sugar chain.

S. aureus, responsible for skin infections, proved sensitive to apigenin-7-*O*-glucoside (**29**), cynaroside (**30**) and pinocembrine (**31**). In this context, cynaroside (**30**) seems to have highly selective properties since it is selectively toxic for bacteria and *T. b. brucei*, while yeast and cancer cells are less sensitive. Further research should be conducted to explain this selectivity. Chrysin (**26**) is another exceptional flavonoid since it is not toxic for bacteria and yeast while being the most toxic flavonoid for *T. b. brucei* and cancer cells. We suggest that the selectivity of flavonoids on their target is much higher than previously expected and thus make flavonoids very interesting lead structures for the development of new drugs.

Our studies established that these extracts contain various phenolic compounds, such as baicalin, baicalein and wogonin (Table 1), which are known for their antioxidative activity.[39,40] The water and methanol extracts showed a high antioxidant activity, 3–5 μg/ml, for all plants. This range is similar to that of the two most active pure compounds, cynaroside (**30**) and scutellarin (**8**) (4 and 13 μg/ml, respectively), while the other flavonoids are around 100 times less active. Minor modifications in the molecules were again responsible for strong variations in their activity.

Conclusions

Our results highlight that two *Scutellaria* species are characterized by a substantial accumulation and a broad structural diversity of flavonoids. The cytotoxicity of chloroform extracts was more potent than other extracts. Especially the chloroform extract of Srr showed a high level of toxicity in all cell lines. Chrysin was the most cytotoxic single substance tested against *T. b. brucei* and in HeLa cells, and was 12 times less toxic than doxorubicin. Especially cynaroside showed strong cytotoxicity in *T. b. brucei*. Among the tested plant chloroform extracts, Srr is a potential source of novel antimicrobial components because of a stronger bactericidal effect on clinically isolated microorganisms, particularly on methicillin-resistant *S. aureus* (MRSA). Also pure flavonoids are in the same range of the tested antibiotics, thus making them promising candidates for lead structures. The water extracts showed high antioxidant activity for all plants, with scutellarin as the most antioxidant substance. Our findings suggest that chloroform extracts and flavonoids of *S. immaculata* and *S. ramosissima* are potentially useful for the development of therapeutic treatments of microbial MRSA infections, trypanosomiasis and cancer.

Declarations

Conflict of interest

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

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