ANTIMICROBIAL AND ANTIOXIDANT COMPOUNDS FROM THE INFUSION AND METHANOLIC EXTRACT OF *Baccharis incarum* (WEDD.) PERKINS.

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ABSTRACT

In the traditional medicine of the South American highlands, the infusion and decoction of aerial parts of "tola", *Baccharis incarum* (Wedd.) Perkins (Asteraceae) is orally taken as an antiseptic, antipyretic and digestive or externally applied to relieve pain and inflammation.

A plant infusion and methanolic (MeOH) extract of the aerial parts were compared for main constituents by HPLC-MS as well as assessed for antimicrobial and free radical scavenging activities. Assay-guided fractionation of the polar extracts was carried out using the $ABTS^{\bullet+}$ autographic as well as methicillin-resistant *Staphylococcus aureus* and *Enterococcus faecalis* bioautographic test. Seven compounds were isolated from the polar extracts and identified as chlorogenic acid, 3',4',5,7-tetrahydroxyflavone; dicaffeoyl quinic acid; 3',4',5,7-tetrahydroxy-3,6-dimethoxyflavone; 3',4',5,7-tetrahydroxy-3,6,8-trimethoxy flavone, 4',5,7-trihydroxy-3',3,6,8-tetramethoxyflavone and 4',5-dihydroxy-3',3,6,7,8-pentamethoxyflavone**.** The main constituents from the methanol extract and infusion were caffeic acid derivatives. This is the first report on the constituents of *B. incarum* infusion and show clear differences with previous phytochemical studies on the same plant. All isolated compounds showed antioxidant activity with SC_{50} values of 1 to 10 µg/ml. The isolated flavones were active against methicillin-resistant *Staphylococcus aureus* and *Enterococcus faecalis* with MICs in the range 100 and >200 µg/ml.

Keywords: *Baccharis incarum*, Asteraceae, infusion, HPLC-MS, polyphenols, antimicrobial activity, antioxidant.

INTRODUCTION

The genus *Baccharis,* with approximately 500 species is one of largest genera of the family Asteraceae. *Baccharis* species are abundant in Argentina where 96 species have been described[1,2]. *Baccharis incarum* (Wedd.) Perkins (syn.: *Baccharis tola* Phil.) known under the popular names lejia, tola and baila buena is common in the arid Andean highlands at 3,800 m above sea level in Antofagasta de la Sierra, Catamarca, Argentina. The infusion and decoction of the aerial parts of *B. incarum* are used in traditional medicine as an antiseptic and antipyretic, gastroprotective, digestive, anti-inflammatory and to relieve muscle and bone pain. The leaves and stems are macerated in ethanol for seven days and the solution is rubbed to relieve rheumatism and inflammation. Plant resin poultices are used in bruises and wounds and to consolidate luxations and broken bones [3-5]. The plant resin has a sweet taste and the bumps in the branches (gills, sometimes called fruit) serve as a substitute for lemon[6]. *B. incarum* grow under conditions of high ultraviolet light, low temperature, high salinity and low atmospheric pressure.

Previous phytochemical studies on the leaves and top parts (aerial parts) of *B. incarum* described the isolation of diterpenes and flavonoids[7-9]. In a screening of medicinal plants from the San Juan Province, Argentine, the antimicrobial effect of *B. incarum* extract on Gram positive and Gram negative bacteria by agar well diffusion method was reported[10]. The xanthine oxidase and antioxidant effect of its ethanolic extracts was described by Zampini *et al*. (2008)[11]. The aim of this work was to assess the antimicrobial activity against multi-resistant clinical isolates and free radical scavenging activity of aqueous and methanolic extracts of aerial parts of *B. incarum* and to identify the main constituents of the traditional crude drug preparation.

EXPERIMENTAL

General

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker spectrometer, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Methanol (CD_3OD) and/or chloroform ($CDCl_3$) were used as solvents. The UV spectra were obtained using a Heλios α V-3.06 UV-VIS spectrophotometer. MeOH was used as solvent. HPLC-DAD was used for the identification and quantification of the main compounds in the crude extracts, fractions and as purity criteria of the isolated compounds before NMR measurements and assays.

HPLC-MS analysis

The determination of phenolics in the samples was carried out as described in previous reports[12,13]. HPLC analysis was performed using HPLC-DAD Merck-Hitachi (LaChrom, USA) equipment consisting of a L-7100 pump, a L-7455 UV diode array detector and D-7000 chromato-integrator. A C18-RP column (Phenomenex, Torrence, CA 5 μm 250 mm x 4.60 mm. i.d.) was used. The compounds were monitored at 254 nm and the absorbance was measured between 200 and 400 nm. Gradient elution was carried out with water-0.1 % formic acid (solvent A) and methanol (solvent B) at a constant flow of 1 ml/min. The solvent composition was as follows. Initial: 10% B, 90 % A, changing to 20% B at 10 min and to 75% B at 45 min, 5 minutes before starting to the initial conditions. Total running time: 50 min.

Mass spectra were measured with Agilent 1100LC equipment connected to an Esquire 4000 LC/MS (Bruker Daltoniks, Germany) system. Extracts and fractions were dissolved in MeOH:formic acid (99:1) (3 mg/ml) for the determinations. Full scan mass spectra were determined between *m/z* 150 and 2000 u in negative mode. Nitrogen at 27.5 psi, 350 ºC and a flow of 8 l/min was used as nebulization gas. Measurement conditions in negative ion mode were as follows: electrospray needle, 4000 v; end plate offset, -500 v; skimmer 1, -56.0 v; skimmer 2, -6.0 v; capillary outlet offset, -84.6 v.

Extract fingerprints and compound identification by HPLC-MS was carried out at a constant flow of 0.5 ml/min. The solvent composition was as follows. Initial: 10% B, 90 % A, changing to 20% B at 20 min and to 75% B at 90 min, 10 minutes before starting to the initial conditions. Total running time: 100 min.

Plant material and extraction

B. incarum (Wedd.) Perkins (synonym: *Baccharis tola* Phil.) was collected in November 2007 in Antofagasta de la Sierra, Catamarca, Argentina at 3,800 m over sea level. The samples were dried in a dark place at room temperature. The parts used were leaves and stems. Voucher specimens were deposited in the Herbarium of "Fundación Miguel Lillo" San Miguel de Tucumán, Tucumán, Argentina and identified by Lic. Soledad Cuello, Fundación Miguel Lillo (Voucher Number 607934 LIL).

The infusion was prepared by placing 200 g of air-dried and ground plant material in some 1l of hot water during 20 minutes. The aqueous extract was liophilized to afford 12 g of solubles (6 % w/w yield). The air-dried and ground plant material (1Kg) was extracted by maceration in methanol (1g/5ml) at room temperature during five days. The methanolic extract (ME) was concentrated at reduced pressure to yield 264 g of solubles (26.4% w/w yield).

Compound isolation

The methanolic extract was resuspended in water (0.5 l) and partitioned with dichloromethane (DCM, 1 l) to afford a DCM-soluble fraction (190 g), an aqueous phase (61.2 g) and a precipitate (PPT, 4 g) in the DCM-water interphase. The aqueous phase and the methanol-soluble precipitate presented similar TLC and HPLC-DAD patterns. The PPT (4 g) was dissolved in MeOH and permeated in a Sephadex LH-20 column (column length 60 cm, internal

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diameter 4.5 cm) using methanol as mobile phase. Some 38 fractions of 50 ml each were collected and pooled according to the TLC analysis (Kieselgel G60 F254 0.2 mm, Merck; mobile phase: ethyl acetate:chloroform 2:1 v/v). The plates were developed with a 0.2% diphenylboric acid ethanolamide complex methanolic solution and the compounds were visualized under UV light at 254 nm and 365 nm[14]. Bioautographic assays were performed to detect antimicrobial compounds. Fractions FR-IV (538 mg) and FR-V (423 mg), active towards *Staphylococcus aureus,* were further purified by permeation on Sephadex LH-20 using methanol as the mobile phase. After TLC comparison, fractions with similar patterns obtained from FR-IV and FR-V were pooled in 7 bioactive groups. The bioactive pool FR2 (168 mg) was submitted to high-speed countercurrent chromatography (HSCCC). The HSCCC instrument (P. C. Inc., Potomac, MD) was equipped with a multilayer coil of 1.68 mm i.d. polytetrafluoroethylene (PTFE) tubing with a total capacity of 180 ml. The revolution radius, or the distance between the holder axis and the central axis of the centrifuge (*R*), was 10.5 cm, and the β value was 0.76 β = *r*/*R*, where *r* is the distance from the coil to the holder shaft. The speed varied between 0 and 1,200 rpm. The flow rate was controlled with a DC Analytic Gearmotor (Bodine Electric Company, Chicago, IL). The solvent system used was *n*-hexane:ethyl acetate: methanol: water 1:1.25:1:1.25; v/v/v/v. This system provided an ideal range of the partition coefficient (*K*) for the applied sample and a desirable settling time (28 s); 66% of the stationary phase was retained in the coil. First, the coiled column was entirely filled with the stationary phase (upper phase, 320 ml). Then, the apparatus was rotated forward at 800 rpm, while the mobile phase (lower phase) was pumped into the column in a head to tail direction at a flow rate of 3.0 ml/min. After the mobile phase front emerged and the hydrodynamic equilibrium was established in the column, about 8 ml of filtered sample solution (dissolved in 4 ml of upper phase and 4 ml of lower phase) was injected through the injection module. The column effluent was continuously monitored by TLC on silica gel. The mobile phase was EtOAc:chloroform 2:1, v/v. The isolation of bioactive compounds is summarized in Fig 1.

Antimicrobial activity

The microorganisms used were clinical samples from *Staphylococcus aureus* (23 isolates) and *Enterococcus faecalis* (3 isolates), obtained from skin and soft tissue infections of patients of Hospital Nicolás Avellaneda, San Miguel de Tucumán, Tucumán, Argentina. The isolates included: MRSA (methicillin-resistant *S. aureus*), MSSA (methicillin-sensitive *S. aureus*), MRCNS (methicillin-resistant coagulase-negative staphylococci), MSCNS (methicillin-sensitive coagulase-negative staphylococci). A reference *S. aureus* strain (ATCC 29213) and *E. faecalis* (ATCC 29212) were included in the study. The strains were identificated by biochemical profiles[15]. All organisms were maintained and the inocula were prepared according to [16]. The number of cells in CAMHB (cation-adjusted Müller Hinton broth) was estimated using a serial dilution technique for each assay[17]. The standard antibiotics: methicillin, vancomycin, oxaciline, gentamicin, ampicillin and streptomycin were used as reference compounds.

Minimal inhibitory concentration (MIC)

MIC values of extract and isolated compounds were determined by bioautographic assay in dot blot and by agar dilution methods[17].

Agar macrodilution method

Serial dilutions (final volume 0.5 ml) of samples were prepared. Then, 4.5 ml of Müller-Hinton agar (MHA) were added. Plates were inoculated with 2 µl of each bacterial cell suspension (5x10^4 CFU) and aerobically incubated for 18 h at 35° C. A growth control of each tested strain was included. MIC_{100} was defined as the lowest concentration of soluble principle or antibiotics at which no colony was observed after incubation.

Bioautographic assays

Plates of silica gel 60 F-254 (0.2 mm, Merck) were seeded in dot blot by duplicate with different fractions or compounds obtained in the purification process. One set of the plates were developed with ethyl acetate:chloroform (2:1, v/v), air-dried and the corresponding duplicate remained in dot blot. Bioautographic assay was performed using 2 ml of soft medium (BHI with 0.6% agar) containing 10^5 CFU of methicillin-resistant *S. aureus* (F7) according to[16]. The growth inhibition areas, yellow coloured, were compared with the Rf of the related spots on the TLC plate revealed with NP.

Total antioxidant capacity assay

Antioxidant capacity assay was carried out by the improved $ABTS^{\bullet+}$ method as described by [18]. $ABTS^{\bullet+}$ radical cation was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate. The antioxidant capacity of the compounds was expressed as SC_{50} (scavenging concentration). All the measurements were conducted in triplicate and results were averaged.

$ABTS^{\bullet+}$ autography

For rapid visualization of free radical scavengers/antioxidants, the compounds were applied on silica gel TLC plates as described in Bioautographic assays. The plates were dried overnight and covered with 3 ml of agar 0.9 % containing 1 ml $ABTS^{\bullet+}$. Plates were incubated at room temperature during 1 minute in the dark. Active samples appeared as light spots against a green-blue background. The antioxidant areas were compared with the Rf of the related spots on the TLC plate revealed with NP [11].

RESULTS AND DISCUSSION

From the aerial parts of *B. incarum* infusion and methanolic extract, several constituents were isolated and/or identified by HPLC-MS and/or spectroscopic methods. A representative HPLC trace of the *B. incarum* infusion and the identification of the main constituents of the water-soluble portion of the MeOH plant extract by HPLC-DAD, LC-MS and LC–MS/MS data are presented in Fig. 2 and Table 1, respectively. According to the HPLC pattern, the main constituents of the plant infusion were chlorogenic acid and dicaffeoylquinic acid.

From the most polar part of the methanolic extract (water-soluble and precipitate), two caffeic acid derivatives and five flavonoids were obtained through assay-guided isolation and identified by spectroscopic and spectrometric means. The fractionation schema is shown in Fig. 1. The ^{1}H NMR, MS and UV of the compounds **1** (3', 4',5,7-tetrahydroxyflavone, luteolin**),** **2** (3',4',5,7-tetrahydroxy-3,6-dimethoxyflavone), **3** (3',4',5,7-tetrahydroxy-3,6,8-trimethoxyflavone), **4** (4',5,7-trihydroxy-3',3,6,8-tetramethoxy flavone) and **5** (4',5 dihydroxy-3',3,6,7,8-pentamethoxyflavone) is in agreement with reported data[19,20] while chlorogenic and dicaffeoylquinic acid were identified by MS-MS, UV spectra and co-chromatography with a standard (chlorogenic acid)[21,22]. The structure of the flavonoids **1-5** is presented in Fig. 3.

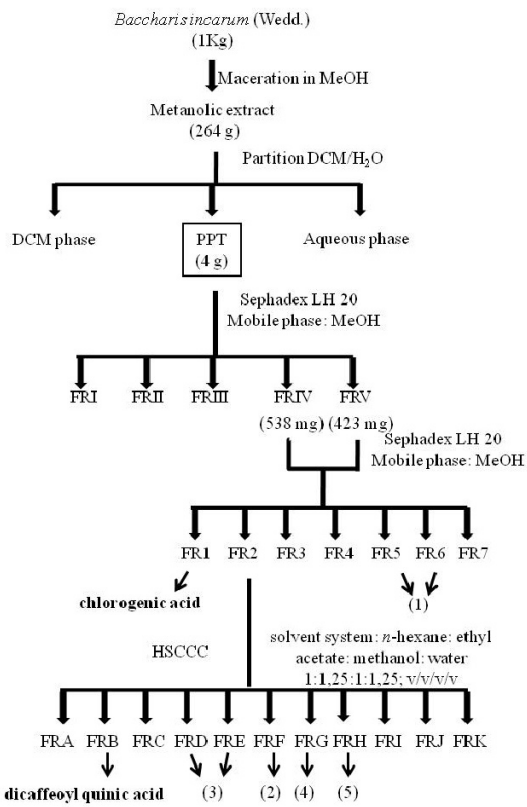


Fig. 1. Fractionation and purification of antibacterial and antioxidant compounds from *B. incarum*.

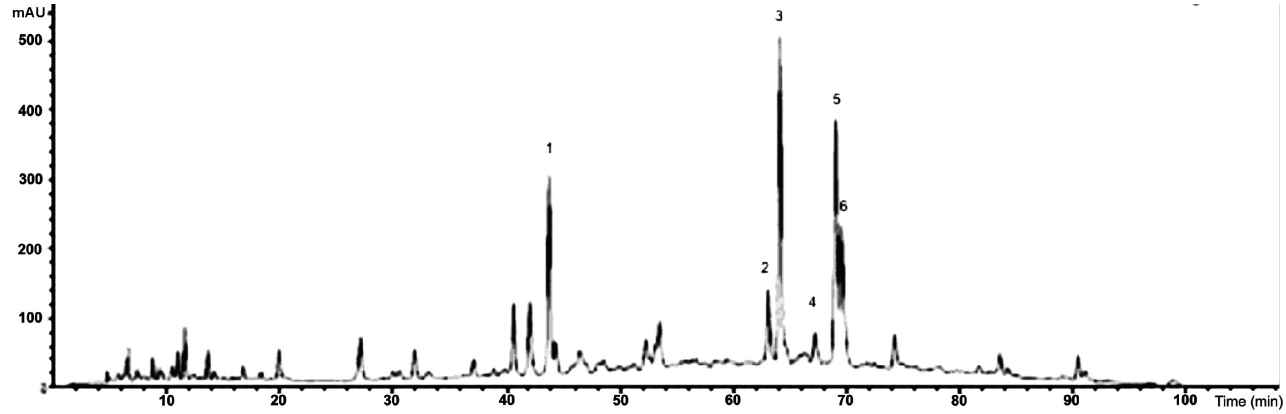
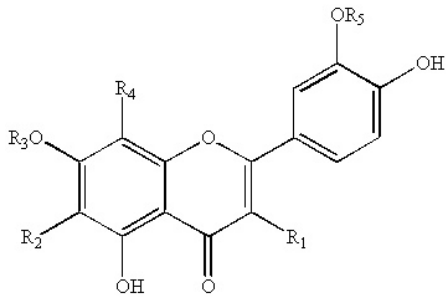


Fig. 2. HPLC trace of *Baccharis incarum* infusion. UV detection: 254 nm.



Compound	R_1	R_2	R_3	R_4	R_5
1: 3',4',5,7-Tetrahydroxyflavone	H	H	H	H	H
2: 3',4',5,7-Tetrahydroxy-3,6-dimethoxyflavone	OCH_3	OCH_3	H	H	H
3: 3',4',5,7-Tetrahydroxy-3,6,8-trimethoxyflavone	OCH_3	OCH_3	H	OCH_3	H
4: 4',5,7-Trihydroxy-3',3,6,8-tetramethoxyflavone	OCH_3	OCH_3	H	OCH_3	CH_3
5: 4',5 Dihydroxy-3',3,6,7,8-pentamethoxyflavone	OCH_3	OCH_3	CH_3	OCH_3	CH_3

Fig. 3. Structure of the flavonoids isolated from the methanolic extract of *B. incarum.*

Table 1. Phenolic compounds in *Baccharis incarum* infusion, water-soluble part of the MeOH extract and precipitate after partition of a total MeOH extract with CH_2Cl_2 as determined by HPLC–DAD, LS–MS and LC–MS/MS data.

Peak	Rt (min)	λ max (nm)	MW	$[M-H]^-$	$[2M-H]^-$	MS/MS ions	Tentative
1	43.6	245, 295 sh, 325	354	353	707	191; 173, 127, 85	Chlorogenic acid
2	63.7	250, 290 sh, 325	742	741		515, 353, 173, 135	-
3	64.2	246, 295 sh, 327	516	515	1031	353, 191, 173, 127	Dicaffeoylquinic acid
4	67.4	255, 344	694	693		517, 193, 175, 160	-
5	69.1	255, 344	610	609		301, 271, 179, 151	Quercetin diglycoside
6	69.7	246, 295 sh, 327	516	515		353, 173, 135	Dicaffeoylquinic acid isomer

The crude methanolic extract was assessed for antimicrobial effect towards several strains of *S. aureus* and *E. faecalis*. The results are summarized in Table 2. The extract presented a relevant activity against both microorganisms, with some selectivity and MICs in the range 40-80 μg/ml. All isolated flavonoids showed moderate to weak activity with MIC values around 100 and >200 μg/ml for *S. aureus* and *E. faecalis* (Table 3). In bioautographic assays, the quantities of isolated flavonoids necessary to inhibit the growth of all strains were 5 μg until 20 μg (Table 3). The results suggest that the antimicrobial effect of the crude drug could be due to the isolated flavonoids and other compounds not identified in this study. The observed activity is in agreement with a previous study on a *B. incarum* sample collected at the Provincia de San Juan in Argentina. The antimicrobial methodology used, however, was agar diffusion and the ethanolic extract presented activity against both Gram positive and Gram negative organisms [10]. These is the first reports of antibacterial activity of *B. incarum* on MRSA, MSSA, MRCNS, MSCNS and *E. faecalis* antibiotic resistant obtained from skin and soft tissue infections of local hospital patients in comparison with American Type Culture Collection (ATCC) strains.

The free radical scavenging activity was determined for *Baccharis* extract and isolated compounds as well as for the synthetic and natural antioxidant (BHT, quercetin). The SC_{50} values were 4 μg/ml and 1-10 μg/ml for the total extract and the isolated compounds, respectively (Table 3). The investigation of the water

infusion and water-soluble portion of the methanol extract of *B. incarum* allowed the identification of caffeic acid derivatives, including chlorogenic acid and dicaffeoylquinic acids as well as flavonoids. The diterpenes previously described in *B. incarum* were not detected in the water infusion, what is not surprising due to the low polarity of those compounds [8,9]. Chlorogenic acid and dicaffeoylquinic acids are know by its antioxidant effect[23-25], antimicrobial[26], antimutagenic[27,28] activities. Five flavones (Fig. 3) were isolated and identified in the present report. Luteolin (compound **1**) has been found to possess antiradical/antioxidative activity, especially when studied in cell-free systems[29], as a glycolytic inhibitor might be a new adjuvant agent for chemotherapy [30], possess antiinflammatory[31], antiespasmodic[32] and antimicrobial activity[33]. Axilarin (compound **2**) was reported in *Artemisia copa* and show antiinflammatory and antioxidant effect as well as inhibition on aldolase activity[34-36]. The compounds **3** and **4** have been previously found in other plant species [37,38] but its biological activity was not reported. The compound **5** (3-methoxycalipterin) was previously isolated from a Chilean collection of *B. incarum*[7]. In a study on the free radical scavenging effect of *B. grisebachii* [38], a strong free radical/antioxidant effect was observed for several flavonoids of this plant. The flavonoids from both *Baccharis* species present a similar substitution pattern with predominance of methoxylated derivatives, consistent with the chemistry of this plant group[38, 39] .

In this work we report for the first time the occurrence of further trihydroxylated and tetrahydroxylated flavones in *B. incarum* as well as caffeoylquinic acid derivatives in the crude drug infusion and methanol extracts. The presence of these compounds can explain at least in part the antioxidant effect of the methanol extract and water infusion of the plant. The study of the water infusion allowed the identification of constituents very different from those previously reported from organic solvents extracts, clearly showing the need to follow the traditional recipes when performing studies on traditional uses of medicinal plants.

Table 2. Antimicrobial activity of *B. incarum* methanolic extracts (ME) and reference antibiotics against *Staphylococcus aureus* and *Enterococcus faecalis* strains.

	MIC_{100} (µg/ml)				
Staphylococcus aureus	Methicillin	Oxacillin	Gentamicin	Vancomycin	*B. incarum* **ME**
Methicillin-resistant					
F1	> 16	> 16	> 100	<1	80
F2, F7	> 16	> 16	> 100	1	80
F31	> 16	> 16	ND	1	80
Methicillin-sensitive					
F4, F6, F9	1	1	< 1	1	80
F13	1	1	< 1	< 1	80
F16, F18, F19	< 1	1	< 1	< 1	80
F32	1	0.5	ND	< 1	40
F24, F26	< 1	1	1	1	80
F28, F34-F38	1	1	ND	< 1	40
Methicillin resistant coagulase- negative					
F22	>16	>16	> 100	<1	80
F27	> 16	> 16	>100	<1	80
Methicillin sensitive coagulase- negative					
F33	1	0.5	ND	< 1	80
F29, F30	1	1	ND	< 1	40
F21	1	1	1	< 1	80
ATCC 29213	< 1	<1	<1	<1	40
Enterococcus faecalis	Ampicillin	Gentamicin	Streptomicin	Vancomycin	*B. incarum* **ME**
F202	1	120	< 300	<6	80
F203	1	> 120	> 300	< 6	80
F226	1	120	> 300	< 6	80
ATCC 29212	1	120	<300	< 1	40

ND: not done

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Table 3. Antimicrobial activity and free radical scavenging activity of *B. incarum* extract and isolated compounds and reference compounds.

Sample	Bioautographic assay * (µg)	MIC* (µg/ml)		$ABTS^{+}$ assay** SC_{50} values µg/ml
	S. aureus	*S. aureus*	*E. faecalis*	
Extract	0.5	40-80	80	4
Compound				
1: 3',4',5,7-Tetrahydroxyflavone	5	100	100	1
2: 3',4',5,7-Tetrahydroxy-3,6-dimethoxyflavone	20	> 100	> 100	6
3: 3',4',5,7-Tetrahydroxy-3,6,8-trimethoxyflavone	20	> 100	> 100	6
4: 4',5,7-Trihydroxy-3',3,6,8-tetramethoxyflavone	5	> 100	> 100	10
5: 4',5 Dihydroxy-3',3,6,7,8-pentamethoxyflavone	10	> 200	> 200	10
Chlorogenic acid	NA	-	-	10
Dicaffeoylquinic acid	NA	-	-	2
Quercetin	-	-	-	2.5
BHT	-	-	-	5

- : not tested
NA: non active until 100 µg/ml
* antimicrobial activity was determined by the bioautographic (dot blot) and agar macrodilution methods (MIC)
** free radical scavenging activity (SC_{50} values) was determined by the ABTS assay

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