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Dihydroisocoumarin from *Xyris pterygoblephara* active against dermatophyte fungi

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Abstract

The ethanol extract from *Xyris pteygoblephara* aerial parts was evaluated against five microorganism strains, by the microdilution and agar diffusion methods. Extract fractionation led to the isolation of three compounds, whose structures were assigned by spectrometric data (1D and 2D NMR, IR, MS and UV) as (3*R*,4*R*)-(−)-6-methoxy-3,4-dihydro-3-*n*-pentil-4-acethoxy-1H-2-benzopyran-1-one (**1**), moronic acid and quercetin. The absolute configuration of **1** was defined by circular dichroism spectroscopy and comparison with data reported for other dihydroisocoumarins. Assay of **1** (100 μg/disc) by the agar diffusion method against clinical isolates of the dermatophytes *Epidermophyton floccosum* (inhibition zone, mm ± s.d.: 4.5 ± 0.8), *Trichophyton mentagrophytes* (4.8 ± 0.4) and *Trichophyton rubrum* (10.2 ± 0.8) revealed similar inhibition zones to the positive control amphotericin B (32 μg/disc; 5.0 ± 0.2; 5.0 ± 0.6 and 8.8 ± 1.2, respectively). The result corroborates the ethnomedical use of *Xyris* species to treat dermatitis.

Keywords: *Xyris pertygoblephara*; Xyridaceae; (3*R*,4*R*)-(−)-6-methoxy-3,4-dihydro-3-*n*-pentil-4-acethoxy-1H-2-benzopyran-1-one; Circular dichroism; Moronic acid; Quercetin; Dermatophyte fungi

1. Introduction

A total of 152 *Xyris* species (Xyridaceae) occurs in Brazil and about 90% of them are endemic (Sajo et al., 1997). *Xyris* plants are small shrubs, popularly known as "sempre-vivas" (everlasting plants). Some are collected for ornamental purposes and for medicinal uses, mainly to treat eczemas and dermatitis (Pio Corrêa and Penna, 1969). In the last years, over exploitation of *Xyris* has contributed to drive some species into a risk of extinction in Brazil.

Both the chemistry and biological activities of *Xyris* are poorly studied. The isocoumarins xyridin A and B were isolated from *Xyris indica* (Ruangrungsi et al., 1995) and the anthraquinones chrysazin and 3-methoxychrysazin were obtained from *Xyris semifuscata* (Fournier et al., 1975). Flavonoids have been described for *Xyris itatiayensis*, *Xyris longiscapa* and *Xyris obtusiuscula* (Varanda et al., 2002). In our previous work, the antifungal activity of seven *Xyris* species has been evaluated and bioguided fractionation of *Xyris pilosa* resulted in the isolation of a new anthraquinone (Cota et al., 2004).

As part of our study directed towards the isolation of biologically active compounds from *Xyris* species, a new antifungal dihydroisocoumarin from *Xyris pterygoblephara* Steud. is here reported, along with two known compounds.

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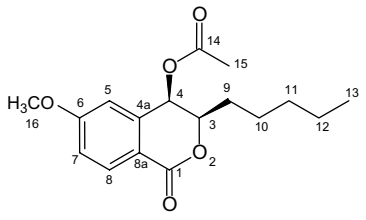
2. Results and discussion

Compound **1** was isolated as a colorless resinous solid. The UV MeOH spectrum of **1** showed absorption maxima at 265.0, 268.8 and 271.6 nm. The IR data confirmed its aromatic character (ν_{max} 1605, 1500 and 1461 cm^{-1}) and established the presence of ester (ν_{max} 1720 cm^{-1}) and aromatic lactone (ν_{max} 1606 cm^{-1}) carbonyl groups. The positive TOF MS spectrum of **1** gave the *quasi*-molecular ion [M+Na] at *m/z* 329.1299, which allowed deducing the molecular formula $C_{17}H_{22}O_5$. The ^{13}C NMR and DEPT-135 spectra indicated three methyls, four methylenes, five methines and five non-hydrogenated carbons, two of them characteristic of carbonyls. The ^{13}C carbonyl carbon signal at δ 164.4 indicated the presence of a lactone moiety whose carbonyl carbon was attached to the aromatic ring at C-8a (δ 117.6) and the lactone oxygen was connected to the C-3 methine carbon (δ 79.3).

The ^1H NMR spectrum showed three aromatic proton signals, comprised *ortho* coupled hydrogens at δ 7.03 (*dd*, $J = 7.2$ and 2.8 Hz, 1H) and δ 8.09 (*d*, $J = 7.2$ Hz, 1H), and of *meta* coupled proton at δ 7.02 ($J = 2.8$ Hz). Correlation of the methyne proton (H-4, *d*, δ 5.87, 1H) to H-3 (*m*, δ 4.53, 1H) and the H-5 aromatic proton (*d*, δ 7.02, $J = 2.8$ Hz, 1H) to the C-4 methyne carbon (δ 67.0), respectively, in the COSY and HMBC experiments, supported a dihydroisocoumarin skeleton (Kongsaeree et al., 2003).

The COSY spectrum also indicated the presence of an *n*-pentyl group, whose position was concluded to be at C-3 due to a correlation of H-3 to H-9 (*m*, δ 1.91, 2H), as well as by HMBC correlations of H-9 to C-3 and C-4. A methoxyl group was suggested by the signal at δ 3.88 (*s*, 3H) and its location at C-6 was defined based on NOE correlations to H-5 and H-7. The presence of an acetoxy group was disclosed by the correlation of a methyl at δ 2.07 (*s*) to the C-14 carbonyl (δ 170.4). The absence of nOe correlation of the methyl protons with aromatic hydrogens confirmed the location of the acetoxy group in the heteronuclear ring at C-4. Conversely, the NOESY spectrum clearly showed cross-peaks between H-3 (δ 4.53) and H-4 (δ 5.87), disclosing the spacial proximity between these hydrogens and therefore their *syn* relationship. This finding was in agreement with the value of $^3J_{3,4} = 2.0$ Hz observed in the ^1H NMR spectrum, consistent with axial-equatorial or equatorial-axial coupling, but incompatible with *trans*-diaxial coupling.



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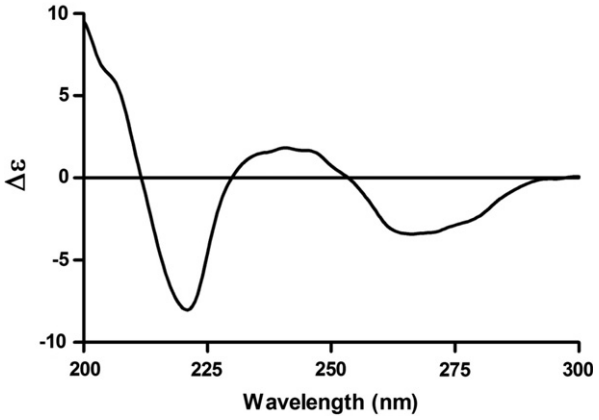

Fig. 1. CD curve of compound (3*R*,4*R*)-(−)-**1** in MeOH.

The absolute configuration of **1** was defined based on analysis of the circular dichroism spectra. The chiroptical properties of the dihydroisocoumarin benzoic ester chromophore has been thoroughly investigated and the sign of the Cotton effect of n→π* origin can be used for establishing the absolute configuration at C-3 (Krohn et al., 1997). Therefore, the CD measurement of **1** was carried out in the UV absorption region in methanol (Fig. 1). The absolute configuration at C-3 was concluded to be *R* based on a negative Cotton effect observed in the CD spectrum of **1**, ascribed to the K-absorption band at 266.5 nm ($\Delta\varepsilon = -3.48$) (Krohn et al., 1997). Taking into account the *syn* relationship between the substituents at C-3 (*n*-pentyl) and C-4 (acetoxy), demonstrated by ^1H NMR and NOESY data, the absolute configuration at C-4 was defined as *R*. On the basis of the above observations, the dihydroisocoumarin structure was established as (3*R*,4*R*)-(−)-6-methoxy-3,4-dihydro-4-acethoxy-5-*n*-pentyl-1H-2-benzopyran-1-one (**1**).

According to the helicity rule for a benzoic ester chromophore, based on the signal of the n→π* transition for dihydroisocoumarin derivatives with a fixed conformation (Antus et al., 1983), the heterocyclic ring of **1** might adopt a semi-chair conformation, which would result in less steric compression for the *n*-pentyl group, located at the pseudo-equatorial position (Fig. 2).

As far as we know, compound **1** has never been described before. The occurrence of dihydroisocoumarins substituted either at C-3 and C-4 is uncommon and among those already reported is monorecin, originally isolated from the fungus species *Helminthosporium monoceras*,

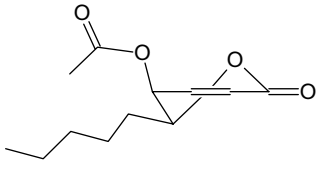

Fig. 2. Standard projection from the aromatic into the heterocyclic ring of (3*R*,4*R*)-(−)-**1** at half-chair conformation (aromatic ring not shown).

Table 1
Antimicrobial activity of *Xyris pterygoblephara* extract, fractions and dihydroisocoumarin **1**, assayed by the microdilution method

IC_{50} (μg/ml)						
Test organisms	Extract	*n*-Hex	CH_2Cl_2	EtOAc	H_2O	**1**
Pseudomonas aeruginosa	>1000	276 ± 30	>1000	623 ± 50	924 ± 82	>500
Staphylococcus aureus	748 ± 46	383 ± 39	n.a.	>1000	>1000	>500
Bacillus subtillis	>1000	920 ± 29	>1000	852 ± 57	878 ± 21	n.a.
Candida albicans	>1000	–	–	806 ± 54	–	–
Aspergillus niger	924 ± 21	>1000	n.a.	>1000	965 ± 19	n.a.

Values are average of six replicates ± standard deviation. (–) no detected activity at the assayed concentrations. n.a., not assayed. Solvents (control) did not show any inhibitory activity. Positive controls included chloramphenicol (*P. aeruginosa* and *S. aureus*), gentamicin (*B. subtillis*) and fluconazole (*C. albicans* and *A. niger*), added in concentrations to produce 100% inhibition (4 μg ml^{-1} for all antibiotics, except fluconazole against *A. niger* which required 64 μg ml^{-1}).

whose absolute configuration was also determined to be 3*R*,4*R* (Grove and Pople, 1979). It should be stressed that this is the first report on the isolation of a dihydroisocoumarin from a Xyridaceae species, although isocoumarins have been previously described for *X. indica* (Fournier et al., 1975; Ruangrungsi et al., 1995). This finding might point out that a compound of this class represents a chemical marker for *Xyris* and a systematic investigation of the genus will be undertaken in the future.

The structures of **2** and **3** were determined by NMR and MS spectroscopy to be the known compounds moronic acid and quercetin, respectively. ^1H and ^{13}C NMR spectroscopic data assignments obtained for **2** and **3** were in accord with published values (Cao et al., 2004; Harborne, 1994). Both compounds are also here described for the first time from a *Xyris* species.

The extract and fractions from *X. pterygoblephara*, along with compound **1**, were initially tested against *Bacillus subtillis* using the agar diffusion method. No inhibition zone was observed in the assayed concentrations (data not shown). Knowing the limitations of the agar diffusion method for testing low hydrophilic matrices (Hostettman et al., 1991), the antimicrobial activity was evaluated by the microdilution method, which additionally allows determining IC_{50} values. As reported in Table 1, the biological activity mainly located in the *n*-hexane fraction, whose major constituent is dihydroisocoumarin **1**, as indicated by TLC and HPLC analysis (data not shown).

Therefore, compound **1** was tested against four strains of dermatophyte fungi, by the agar diffusion method. It was not feasible to carry out the assays by the microdilution method due to the limited solubility of amphoterincin B (positive control) in aqueous medium. Alternatively, fluconazole was evaluated as positive control but fungal resistance impaired its use. Compound **1** showed significant antifungal activity and produced inhibition zones comparable to those of amphotericin B (Table 2).

Table 2
Antifungal activity of dihydroisocoumarin **1** against dermatophyte isolates, assayed by the agar diffusion method

Fungal inhibition zone (mm diameter zone ± sd)		
Test organisms	**1**	Amphotericin B
Epidermophyton floccosum	4.5 ± 0.8	5.0 ± 0.2
Mycrosporum canis	–	12.7 ± 1.2
Trichophyton rubrum	10.2 ± 0.8	8.8 ± 1.2
Trichophyton mentagrophytes	4.8 ± 0.4	5.0 ± 0.6

Values are average of six replicates ± standard deviation. (–) no detected activity at the assayed concentrations. Solvent (CH_2Cl_2) did not show any inhibitory activity.

3. Concluding remarks

Several biological activities were previously described for dihydroisocoumarins isolated from fungi, including antifungal properties (Kongsaeree et al., 2003). Synthetic dihydroisocoumarins have been also described to be active against *Trichophyton mentagrophytes* (Saeed, 2003). Nevertheless, as far as we know this is the first report of a plant derived dihydroisocoumarin active against dermatophytic fungi. The result is of special relevance, since it was obtained using clinical isolates, which are more prone to resistance. These data are clear evidence to support the ethnomedical use of *Xyris* species to treat dermatitis.

4. Experimental

4.1. General

^1H NMR (400 MHz), ^{13}C NMR (100 MHz), NOESY, HMQC, and HMBC spectra were obtained in $CDCl_3$ with TMS as internal standard and were recorded on a Bruker Avance DRX-400 equipment. UV spectra were recorded in MeOH on a UV/Visible Perkin–Elmer model Lambda 20 spectrophotometer and IR spectra in $CHCl_3$ on a Shimadzu IR-400 spectrophotometer. Silica gel (Merck 230–400 mesh) and Sephadex LH-20 (Amersham Biosciences) were used for CC separation while silica gel 60 (Merck) was used for analytical TLC (0.25 mm). Preparative HPLC was carried out on a Shimadzu system (Japan) composed of pump LC-8A, UV–VIS detector SPD-GAV, controller system SCL-8A and integrator C-R4A. A silica gel column (Shim-pack prep sil 250 × 20 mm i.d., Shimadzu, Japan) was employed.

4.2. Plant material

Aerial parts of *X. pterygoblephara* Steud. were collected in Minas Gerais state, Brazil, in March 2003, at Serra do Cipó National Park (coordinates: 19°14′51″S, 43°30′42″W; alt.: 1320 m). The species was identified by botanists from Fundação Zoo-Botânica, Belo Horizonte, Brazil, where a voucher specimen is preserved (BHZB 2496).

4.3. Extraction and isolation

The dried capitula and scapes of *X. pterygoblephara* (48 g) were extracted by percolation with EtOH–H_2O (96:4, v/v). The solvent was removed in vacuo in a rotatory evaporator at 50 °C, affording a dark residue (7.1 g). The extract was submitted to two distinct fractionation procedures. In the first, a portion (5.0 g) was sonicated for 20 min with CH_2Cl_2 (3 × 20 ml), the solvent was eliminated and afforded the "soluble residue" (1.7 g). In this sequence, the residue was subjected to silica gel CC with *n*-hexane, *n*-hexane–CH_2Cl_2 (9:1, 1:1, 1:9), CH_2Cl_2–EtOAc (9:1, 8:2, 7:3, 6:4, 1:1), EtOAc–MeOH (1:1, 2:8) and MeOH successively as eluants to give 12 fractions. The CH_2Cl_2–EtOAc (9:1) eluate (528.9 mg) was sequentially purified by flash chromatography on silica gel columns eluted with CH_2Cl_2, CH_2Cl_2–EtOAc (7:3) and *n*-hexane–acetone (9:1) to afford (3*R*,4*R*)-(−)-6-methoxy-3,4-dihydro-3-*n*-pentil-4-acethoxy-1H-2-benzopyran-1-one (**1**) (355.7 mg).

The dichloromethane "insoluble residue" (3.2 g) was fractionated by silica gel CC eluted with *n*-hexane–CH_2Cl_2 (8:2, 6:4, 1:1, 4:6, 3:7, 1:9), CH_2Cl_2, CH_2Cl_2–EtOAc (9:1, 8:2, 6:4, 1:1, 2:8), EtOAc, EtOAc–MeOH (9:1, 8:2, 7:3, 6:4, 4:6, 2:8, 1:9) and MeOH to originate 24 fractions. The fraction eluted with EtOAc–MeOH (7:3) (730.8 mg) was sequentially applied to silica gel (eluent: EtOAc–formic acid–HAc–MeOH 100:11:11:27) and Sephadex LH20 (eluent: MeOH) columns to obtain quercetin (**2**) (5.6 mg).

In the second procedure adopted for extract fractionation, a portion (2 g) was suspended in MeOH–H_2O (1:1; 120 ml) and sequentially partitioned with equal volumes (3 × 40 ml) of *n*-hexane, CH_2Cl_2 and EtOAc. The MeOH was removed in vacuo before partitioning the extract suspension with CH_2Cl_2 and EtOAc. Solvents were eliminated in a rotatory evaporator, at maximum temperature of 50 °C. The *n*-hexane fraction was further subjected to silica gel column chromatography eluted with *n*-hexane, *n*-hexane–CH_2Cl_2 (85:15, 8:2, 75:25, 7:3, 65:35, 1:9, 5:95), CH_2Cl_2 and CH_2Cl_2–EtOAc (95:5, 9:1, 55:45, 25:75, 5:95). The eluate CH_2Cl_2–EtOAc (55:45) (44.1 mg) was additionally purified by HPLC on a silica gel column using *n*-hexane–CH_2Cl_2 (5:95) as eluent to give moronic acid (**3**) (10.2 mg).

4.4. (3R,4R)-(−)-6-methoxy-3,4-dihydro-3-n-pentyl-4-acethoxy-1H-2-benzopyran-1-one (1)

Colorless resinous solid, $[\alpha]_D^{25}$ − 66 ($CHCl_3$, *c* 0.0326). UV λ_{max} nm: 265.0, 268.8, 271.6 nm; IR ν_{max} cm^{-1}: 2954, 2932, 2861, 1720, 1605, 1500, 1461, 1267, 1219, 1116, 1093; TOF MS (positive ion): found *m*/*z* 329.1299 [M+Na]; calculated for $C_{17}H_{22}O_5$ *m*/*z* 306.1513; ^1H NMR spectral data (400 MHz, $CDCl_3$): δ 8.09 (1H, *d*, *J* = 7.2 Hz, H-8), 7.03 (1H, *dd*, *J* = 7.2, 2.8 Hz, H-7), 7.02 (1H, *d*, *J* = 2.8 Hz, H-5), 5.87 (1H, *d*, *J* = 2.0 Hz, H-4), 4.53 (1H, *m*, H-3), 3.88 (1H, *s*, H-16), 2.07 (1H, *s*, H-15), 1.91 (2H, *m*, H-9), 1.70 (1H, *m*, H-10), 1.35 (2H, *m*, H-11, H-12), 0.90 (1H, *t*, *J* = 7.2 Hz, H-13); ^{13}C NMR spectral data (100 MHz, $CDCl_3$): 164.44 (C-1), 79.29 (C-3), 67.03 (C-4), 138.69 (C-4a), 113.29 (C-5), 163.97(C-6), 116.30 (C-7), 132.54 (C-8), 117.63 (C-8a), 31.47 (C-9), 24.59 (C-10), 30.51 (C-11), 22.43 (C-12), 13.95 (C-13), 170.43 (C-14), 20.84 (C-15), 55.72 (C-16); CD $\Delta\varepsilon$ −8.11 (221.5 nm), +1.90 (240 nm), −3.48 (266.5 nm) (*c* 0.02 mg ml^{-1}, methanol).

4.5. Determination of absolute configuration of 1

Absolute configuration was determined by circular dichroism spectra recorded on a JASCO J-720 spectropolarimeter (Jasco Inc., Easton, USA), equipped with a Peltier type temperature controller. The instrument was calibrated using (+)-10-camphorsulfonic acid (Sigma–Aldrich, St. Louis, USA) (Chen and Yang, 1977). Spectra were obtained from 200 to 300 nm at 25 °C using 0.1 cm path length cells. Compound **1** was dissolved in methanol (Mallinckrodt Baker Inc., Phillipsburg, USA) to a final concentration of 0.02 mg ml^{-1}. The solution was stirred and incubated at room temperature for 10 min before each spectrum was recorded. The spectra were an average of eight scans recorded at a speed of 10 nm min^{-1}, with a bandwidth of 1.0 at 0.5 nm step size and a 2 s time constant. After background subtraction and smoothing, CD data was expressed in molar circular dichroism units. The software Spectra Manager (Jasco) was used for data collection and analysis.

4.6. Microbial cultures and growth conditions

B. subtillis (ATCC 19659), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 25619), *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 16404), *Epidermophyton floccosum*, *Mycrosporum canis*, *T. mentagrophytes* and *Trichophyton rubrum* were used as test microorganisms. Strains of the dermatophyte fungi (*E. floccosum*, *T. mentagrophytes*, *M. canis*, and *T. rubrum*) were isolated from patients of Hospital Socor, Belo Horizonte, Brazil, in February 2006 and were donated by Pharm. Fabrizio Resende Guglielmeli.

The cultures of fungi and bacteria were grown in PDA and agar medium, respectively, in tubes kept in a slanting position, at room temperature (22–25 °C) for 4–5 days, and at 36 °C, for 24 h. Cultures were maintained in plates, at 4 °C, in Sabouraud agar for fungi, and in no. 1 antibiotic agar for bacteria.

4.7. Agar diffusion assay

The antibacterial activity of *X. pterygoblephara* extract, fractions (*n*-hexane, CH_2Cl_2, EtOAc and water) and compound **1** was evaluated by the agar diffusion method against *B. subtillis*. This method was also employed for assaying the activity of **1** against the dermatophyte fungi (*E. floccosum*, *T. mentagrophytes, M. canis,* and *T. rubrum*).

For the assays, sample solutions were prepared in appropriate solvents (MeOH for the extract, *n*-hexane, CH_2Cl_2, EtOAc and water for the respective fractions and CH_2Cl_2 for **1**) to concentrations of 100, 50 and 5 mg ml^{-1}, respectively. Suspensions of microorganisms were prepared in peptone saline solution. The transmittance of the inoculum suspension was adjusted to $50 \pm 1\%$, at 580 nm. Seeded agar plates were prepared by pouring 20 ml of no. 1 antibiotic agar into each plate. After medium solidification, each plate was overlaid with 5 ml medium containing 0.05% of the inoculum suspension. Sterile paper discs (6 mm diameter) were impregnated with 20 μl of the extract (2000 μg/disc), fractions (1000 μg/disc) or compound **1** (100 μg/disc). The discs were placed in duplicate onto the plates and incubated for 24 h, at 37 °C for *B. subtillis*, for 5 days, at 30 °C for *T. mentagrophytes, T. rubrum* and *E. floccosum*, and for 12 days, at 30 °C for *M. canis*. The experiments were carried out in six replicates. The results (mean value plus standard deviation) were recorded by measuring the zones of growth inhibition surrounding the discs. Chloramphenicol (3 μg/disc) and amphotericin B (32 μg/disc) were included in the assays as positive control, whereas control disks contained solvents only as negative control.

4.8. Microdilution assay

A broth microdilution method was used to determine the 50% inhibitory concentration (IC_{50}) of *X. pterygoblephara* extract, fractions and dihydroisocoumarin **1** against *B. subtillis*, *S. aureus*, *P. aeruginosa*, *C. albicans* and *A. niger*. Due to the limited mass of the CH_2Cl_2 fraction, it was assayed solely against the three first microorganisms. Samples were suspended in aqueous Tween 80 (1% v/v) to concentrations of 2000 μg ml^{-1} (extract and fractions) or 1000 μg ml^{-1} (compound **1**) and were sterilized by UV light for 2 h, before the assays. Suspensions of microorganisms were prepared as described for the agar diffusion assay.

For the assays, serial dilutions of the sample solutions were prepared in a microtitre plate over the range 1000–50 μg ml^{-1} for extract and fractions, and 500–25 μg ml^{-1} for compound **1**. In the sequence, the microorganism suspension (5 μl) was added and aqueous Tween 80 (1% v/v) was supplemented to each well up to 200 μl. Positive and negative growth controls were included in every test. The plate was incubated aerobically for 24 h at 37 °C (bacteria and fungi) or for 48 h at 36 °C (*A. niger*). After the incubation period, 20 μl of MTT aqueous solution (5 mg ml^{-1}) was added to each well, following agitation (10 min, 200 rpm) and incubation for 1 h at room temperature. Finally, absorbance was read in a microtitre plate reader at 595 nm. All tests were performed in Mueller–Hinton Broth (Merck, Germany) in triplicate.

The antimicrobial activity, expressed as inhibition percentage, was calculated using the following expression:

$$\% \text{ inhibition} = \frac{\text{Ac} \cdot \text{At}}{\text{Ac}},$$

where Ac is the measured absorbance at 595 nm for the positive control and At is the sample absorbance subtracted the blank absorption.

IC_{50} was calculated from concentration-inhibition curves (GraphPad Prism 3, USA).

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