Bioactivity of the compounds isolated from
Blepharocalyx salicifolius

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Abstract: Blepharocalyx salicifolius (Kunth) O. Berg, Myrtaceae, is an endemic
species that occurs at Southern America. This species was studied to intend
isolation of the active compounds that could be used in vitro model against
leishmaniosis, tumoral cell and paracoccidioidomycosis. After Gel Permeation
Chromatography, the ethanolic extract from leaves yielded sixteen fractions. Five
compounds were isolated and assayed, showing activity against tumoral cells, from
3.33 to 12.83 μg.mL⁻¹; Leishmania (Leishmania) amazonensis from 2.19 to 20.80
μg.mL⁻¹ and Paracoccidioides brasiliensis from 3.10 to 12.5 μg.mL⁻¹.

Keywords: biological assays
Blepharocalyx salicifolius
chalcone
flavonoid
Myrtaceae
triterpene

Introduction

Blepharocalyx salicifolius (Kunth) O. Berg, Myrtaceae, is an endemic species that occurs at
Southern America, especially in countries like Brazil, Paraguay, Argentina and Uruguay. It is a bush that
grows until 15 m length. The popular culture has presented several therapeutic indications for this
species (Mors et al., 2000). Biological activities, by means of infusion from leaves, have been described
as antibacterial (with Staphylococcus aureus and Echerichia coli), anti-inflammatory, antinociceptive,
antispasmodic and intestinal transit models (Limberger et al., 2001). Five compounds were isolated and assayed
against the pathogenic agents of the leishmaniasis, paracoccidioidomycosis and additionally against
human cancer cell lines. It is necessary to improve the therapeutic arsenal against these diseases because the
actual drugs are insufficient, very toxic or the parasites have presented resistance (Pecoul et al., 1999). This
work is part of the ongoing research project for this species, in order to discover compounds, from natural
source, which can be used for this purpose. (Siqueira et al., 2010).

Material and Methods

General

Gel Permeation Chromatography (GPC) was carried out using Sephadex™ LH-20 gel (GE Healthcare,
USA). Ethanol was used as mobile phase. Thin layer chromatography (TLC) was developed using silica
HF₂₅₄ plates (Merck). The spots were visualized after spraying the plates with vanillin-sulfuric acid or NP/PEG
(diphenylborinic acid ethanolamine ester - polyethylene glycol). Adsorptive column chromatography (ACC) was
carried out using silica-gel 60-230 mesh (Merck) or Silica-
gel HF60 (Merck). Reverse Phase HPLC (RP-HPLC) was
carried out by Shimadzu HPLC System, using a Shim-
pack® C18 column (5 μm, 250 mm x 20 mm i.d.). Gas
Chromatography/Mass Spectrometry (GC/MS) analyses
were performed on a Shimadzu QP-5050A instrument,
equipped with a with a PTE™-5 column (30 m, 0.25 mm,
0.25 μm, Supelco, USA), using helium as the carrier gas.
Nuclear Magnetic Resonance (NMR) experiments (1H and
13C, DEPT, HMOC, HMBC and COSY) were recorded
on a Bruker DRX 400 spectrometer using standard
Bruker pulse sequences and appropriate solvents. Electron
impact (70 eV) low-resolution mass spectra (EI-MS) were
obtained by means of direct injection on a Shimadzu
QP5050A device.
Bioactivity of the compounds isolated from *Blepharocalyx salicifolius*

Ezequias P. Siqueira et al.

**Crude extract**

Leaves of *Blepharocalyx salicifolius* (Kunth) O. Berg, Myrtaceae, species were collected at Parque Estadual do Rio Preto, Minas Gerais State, Brazil, in November 2006. Exsiccates of the species were committed at UFMG Herbarium (BHCB 87484). Crude extract was obtained by means of maceration of the *B. salicifolius* leaves. The material was dried using an oven at 35 °C for two weeks. The leaves were grounded and conditioned in ethanol (analytical grade). Extraction process was performed three times. The raw extract was obtained after filtration and evaporation to dryness, using a rotary evaporator.

**Isolation of the compounds**

Raw extract (about 3.74 g) was dissolved in ethanol (20 mL) and injected in the GPC system. It was obtained 190 fractions that were grouped by means of TLC, according to chemical profile, using dichloromethane/methanol (95/5 v/v) as eluent. The fractions were collected and analyzed by HPLC, using methanol/water (150 mg) was dissolved in 10 mL methanol/water (1/1 v/v) and purified by RP-HPLC, using methanol/water gradient as eluent.

The F5 fraction (200 mg) was partitioned between methanol and n-hexane three times. The methanolic phase was partitioned sequentially, using a mixture of n-hexane/ethyl acetate (70/30 v/v), until complete exhaustion of methanolic phase. After evaporation of the solvent, this material was purified by ACC, using isocratic hexane/ethyl acetate (70/30 v/v) as eluent. The fractions were collected and analyzed by TLC, yielding compound 5 (17 mg).

**Compound 1**: ¹H NMR (400 MHz, CDCl₃) δ 13.63 (s, 1H), 7.98 (d, 1H, J = 16.0 Hz), 7.83 (d, 1H, J = 16.0 Hz), 7.64-7.61 (m, 2H), 7.42-7.37 (m, 3H), 5.70 (s, 1H), 3.65 (s, 3H), 2.15 (s, 3H), 2.13 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 193.4, 162.0, 159.4, 158.8, 142.9, 135.3, 130.2, 128.9, 128.4, 126.7, 109.0, 109.1, 106.7, 62.3, 8.2, 7.5. MS/El (M⁺) 298.

**Compound 2**: ¹H NMR (400 MHz, CDCl₃) δ 13.09 (s, 1H), 7.97 (d, 1H, J = 16.0 Hz), 7.86 (d, 1H, J = 16.0 Hz), 7.66-7.63 (m, 2H), 7.42-7.39 (m, 3H), 3.75 (s, 3H), 3.66 (s, 3H), 2.17 (s, 3H), 2.16 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 194.1, 163.7, 161.7, 158.7, 143.4, 135.3, 130.4, 128.9, 128.5, 126.5, 115.7, 115.6, 111.9, 62.3, 60.1, 8.8, 8.7. MS/El (M⁺) 312.

**Compound 3**: ¹H NMR (400 MHz, CDOD) δ 7.37-7.32 (m, 2H), 6.94 (d, 1H, J = 8.0 Hz), 6.40 (d, 1H, J = 2.0 Hz), 6.23 (d, 1H, J = 2.0 Hz), 5.38 (d, 1H, J = 1.6 Hz), 4.30 (dd, 1H, J = 3.3 and 1.6 Hz), 3.78 (dd, 1H, J = 9.0 and 3.3 Hz), 3.45-3.37 (m, 2H), 0.97 (d, 3H, J = 6.0 Hz). ¹³C NMR (100 MHz, CDOD) δ 179.8, 166.0, 163.4, 159.5, 158.7, 150.0, 146.6, 136.4, 123.1, 123.0, 117.1, 116.5, 103.7, 100.0, 94.8, 73.4, 72.3, 72.2, 72.0, 17.8. MS/El (M⁺) 448.

**Compound 4**: ¹H NMR (400 MHz, CDOD) δ 7.49-7.44 (m, 2H), 6.86 (d, 1H, J = 8.0 Hz), 6.36 (d, 1H, J = 2.0 Hz), 6.17 (d, 1H, J = 2.8 Hz), 5.43 (bs, 1H), 4.29 (dd, 1H, J = 2.8 and 1.0 Hz), 3.85-3.75 (m, 2H), 3.41-3.38 (m, 2H). ¹³C NMR (100 MHz, CDOD) δ 180.2, 166.2, 163.3, 159.5, 158.7, 150.0, 146.5, 135.1, 123.3, 123.2, 117.0, 116.6, 109.7, 100.0, 94.9, 88.2, 83.5, 78.9, 62.7. MS/El (M⁺) 434.

**Compound 5**: ¹H NMR (400 MHz, CDOD) δ 5.23 (s, 1H), 3.21 (dd, 1H, J = 9.5 and 5.2 Hz); 2.28 (d, 1H, J = 11.1 Hz), 1.98 (dd, 2H, J = 13.7 and 3.5 Hz), 1.64 (m, 2H); 1.63 (s, 1H); 1.61 (m, 2H); 1.60 (m, 2H), 1.57 (m, 1H), 1.51 (m, 1H), 1.48 (m, 2H), 1.36 (m, 1H), 1.35 (m, 1H), 1.34 (m, 2H), 1.31 (m, 3H), 1.09 (m, 2H), 0.95 (d, 3H, J = 6.8 Hz), 0.93 (s, 3H), 0.89 (s, 3H), 0.85 (d, 3H, J = 5.9 Hz); 0.71 (s, 3H), 0.70 (s, 3H), 0.69 (s, 1H). ¹³C-NMR (100 MHz, CDOD): δ 179.0, 138.3, 124.9, 78.3, 77.2, 55.1, 52.7, 47.4, 41.8, 39.2, 38.9, 38.7, 38.6, 38.5, 36.7, 36.7, 32.8, 30.6, 29.4, 28.0, 28.1, 27.0, 24.2, 23.3, 23.0, 21.0, 18.1, 16.8, 16.7, 15.5, 15.1. MS/El (M⁺) 456.

**Biological Assays**

**Cytotoxicity assays with human cancer cell lineages**

The cytotoxicity assays were performed using the tumor cell lineages UACC-62 (human melanoma), MCF-7 (human breast cancer) and TK-10 (human renal cancer). The cell lineages were purchased from the National Cancer Institute (NCI, Maryland, USA). The cell toxicity assays were run according to the protocols established at NCI, using the sulphorhodamine colorimetric assay (Monks et al., 1991). Results were expressed in terms of the growth inhibition percentage (%), where the sample tested was considered cytostatic...
from 0-99% and cytotoxic from 100-200%.

Assays using amastigotes-like of Leishmania (Leishmania) amazonensis

Promastigotes of L. (L.) amazonensis (strain IFLA/BR/196/PH-8) were obtained from lesions of infected hamsters. The assays were run according to Teixeira et al. (2002). The results were expressed as percent of inhibition in relation to the controls without drug.

Culture and maintenance of Paracoccidioides brasiliensis

Eleven clinical P. brasiliensis strains, Pb-01 (ATCC- MYA-826), Pb-18 (Fungi Collection of the Faculty of Medicine of the Universidade de São Paulo, São Paulo, SP, Brazil), Pb-B339 (ATCC 32069), Pb-14 (clinical isolate from acute PCM, São Paulo, Brazil), Pb-3 and Pb-4 (clinical isolates from chronic PCM, São Paulo, Brazil– MHH Forjaz/TIE Svidzinski), Pb-2 (Epm 60), Pb-1578, Pb-ED01, Pb-11, Pb-8 (clinical isolates from acute PCM, Paraná, Brazil, TIE Svidzinski) were used in the biological assays. P. brasiliensis strains were maintained according to the CLSI document M 27-A2 (NCCLS, 2002).

Determination of the Minimal Inhibitory Concentrations (MIC)

Susceptibility was determined by the microbroth dilution method. Broth microdilution test was performed in accordance with the guidelines in the CLSI document M27-A2 (NCCLS, 2002) and modifications suggested by Johann et al. (2010).

Determination of the Minimal Fungicidal Concentrations (MFC)

The MFC values for pure compounds were determined according to Espinel-Ingroff et al. (2001) and Portillo et al. (2005).

Results and Discussion

Isolated compounds

The structures of the compounds were established by their 1D and 2D NMR spectroscopic data and comparison with literature data. The compound 1 was identified as (2E)-1-(2',4'-dihydroxy-6'-methoxy-3',5'-dimethylphenyl)-3-phenylprop-2-en-1-one, a chalcone isolated in other species (Malterud et al., 1977; Gonzales et al., 1992; Gafner et al., 1996). The compound 2, (2E)-1-(4'-hydroxy-2',6'-dimethoxy-3',5'-dimethylphenyl)-3-phenylprop-2-en-1-one, showed similar structure to 1, seems to be an unedited substance. The compounds 3-4, isolated as pale yellow solids, were elucidated by means of NMR data, hydrolysis, peracetylation and GC/MS analysis (Cota et al., 2008; Monteiro et al., 2009; Monteiro et al., 2010). It was possible to confirm the identity of the sugar moiety as rhamnose for 3 (Figure 1) and arabinose for 4 (Figure 2). Pure quercitrin, purchased from Sigma-Aldrich, has corroborated our elucidation for 3. This flavonoid is a usual substance, isolated from several species (Hallet & Parks 1951; Lopez, 1982; Kato et al., 2010). The compound 4 had similar aglyconic molecular structure to 3 thus, the substance was elucidated as guaijaverin, a flavonoid found in several species (Mair et al., 1987; Arisawa et al., 1993; Xu et al., 2009). By means of spectroscopic data and literature (Seebacher et al., 2003; Silva et al., 2008), it was possible to confirm the identity of the 5 as ursolic acid, (3β-hydroxy-urs-12-en-28-oic acid), an ursanic triterpene isolated from several species (Fu et al., 2005; Leite et al., 2006; Cunha et al., 2007).

These five compounds are reported herein for the first time for this genus and species. Previous report about this genus has described GC-MS analysis of the essential oils, where the principal identified compounds were monoterpenes and sesquiterpenes (Limberger et al., 2001).

Biological assays

All compounds were evaluated against three human tumor cell lineages: UACC-62 (melanoma), MCF-7 (breast cancer) and TK-10 (renal cancer), against amastigotes-like of L. (L.) amazonensis and P. brasiliensis. The results, (Table 1), demonstrated that only chalcones (1-2) had activity for all biological assays. They exhibited antifungal activity against all
isolates of *P. brasiliensis*, with MIC values ranging from 3.1 to 12.5 μg.mL⁻¹. The results for MFC activity for 1 were two concentration orders above MIC results for Pb-18 and Pb-B339 (3.1 and 12.5 μg.mL⁻¹, for both strains, respectively). Compound 2 demonstrated MFC results one concentration order above MIC for Pb-18, Pb-B339, Pb-01 and Pb-1578 strains (3.1 and 6.2 μg.mL⁻¹, for all strains, respectively). Although antifungal activity has been related to chalcones, this is the first work that describes fungicide activity for these compounds against *P. brasiliensis*. Chalcones 1 and 2 exhibited the best IC50 values against amastigotes-like of *L. (L.) amazonensis* between the five isolated compounds (5.23 and 2.19 μg.mL⁻¹, respectively). Torre-Santos et al. (1999) showed an IC50 of 24 μg.mL⁻¹ against intracellular amastigotes of *L. (L.) amazonensis* for structurally similar chalcone, 2',6'-dihydroxy-4'-methoxychalcone, isolated from dichloromethane extract of *Piper aduncum* inflorescences. Chalcones 1 and 2 were active against all cancer cell lineages tested. They presented values of IC50 ranging from 3.00 to 10.83 μg.mL⁻¹ and MCF-7 lineage was the most sensitive to these compounds. The literature reports scarce biological activity for chalcone 1. It was noticed antimicrobial activity against *Staphylococcus aureus* (MIC of 250 μg.mL⁻¹) (Belofsky et al., 2004) and *Mycobacterium tuberculosis* (MIC of 62.5 μg.mL⁻¹) (Pavan et al., 2009) and it was not reported any biological activity for chalcone 2.

Quercitrin (3) and guaijaverin (4) exhibited only leishmanicidal activity, with IC50 values of 6.25 and 20.80 μg.mL⁻¹, respectively. The kind of sugar linked to aglycone moiety on flavonoids affects its activity. According to Muzitano et al. (2006) quercitin, isolated from aqueous leaf's extract from *Kalanchoe pinnata*, had an IC50 of 8 μg.mL⁻¹ and the presence of quercetin aglycone and the sugars linked to, suggests the importance of this structural feature for antileishmanial activity. Aglycon quercetin, together with five of its glycosides, which includes quercitin and guaijaverin, were isolated from *Psidium guajava* L. This species is used against diarrhea and the results demonstrated that the ability to inhibit peristalsis is mainly due to the aglycon quercetin (Lozoya et al., 1994).

Ursolic acid is a ubiquitous pentacyclic triterpenoid in plant kingdom (Liu, 2005). Our results demonstrated that this compound was had cytotoxic against all cell lineages tested (IC50 ranging from 10.83 to 12.83 μg.mL⁻¹). This compound has showed...
Table 1. Results of the biological assays expressed in concentration of the compound (µg.mL\(^{-1}\)) sufficient to inhibit the growth or to kill the pathogenic agents.

<table>
<thead>
<tr>
<th>Pathogenic agent</th>
<th>Compounds</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK-10(c)</td>
<td>4.33±0.58</td>
<td>10.83±1.44</td>
<td>Not active</td>
<td>Not active</td>
<td>12.83±3.01</td>
<td>16(d)</td>
<td></td>
</tr>
<tr>
<td>UACC(a)</td>
<td>5.00±0.10</td>
<td>7.00±1.73</td>
<td>Not active</td>
<td>Not active</td>
<td>10.83±1.44</td>
<td>16(e)</td>
<td></td>
</tr>
<tr>
<td>MCF-7(a)</td>
<td>3.33±0.57</td>
<td>3.00±1.80</td>
<td>Not active</td>
<td>Not active</td>
<td>11.67±1.44</td>
<td>16(f)</td>
<td></td>
</tr>
<tr>
<td><em>L. (L.) amazonensis</em>(b)</td>
<td>5.23±1.84</td>
<td>2.19±1.60</td>
<td>6.25±0.10</td>
<td>20.80±7.21</td>
<td>9.34±6.80</td>
<td>0.20(g)</td>
<td></td>
</tr>
<tr>
<td>Pb-01(c)</td>
<td>6.20/6.20</td>
<td>3.10/6.20</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>300/300</td>
<td></td>
</tr>
<tr>
<td>Pb-18(c)</td>
<td>3.10/12.50</td>
<td>3.10/6.20</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>300/300</td>
<td></td>
</tr>
<tr>
<td>Pb-B339(c)</td>
<td>3.100/12.50</td>
<td>3.10/6.20</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>150/150</td>
<td></td>
</tr>
<tr>
<td>Pb-14(c)</td>
<td>6.20/6.20</td>
<td>6.20/6.20</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>300/300</td>
<td></td>
</tr>
<tr>
<td>Pb-3(c)</td>
<td>6.20/6.20</td>
<td>6.20/6.20</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>150/150</td>
<td></td>
</tr>
<tr>
<td>Pb-4(c)</td>
<td>12.50/12.50</td>
<td>12.50/12.50</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>150/150</td>
<td></td>
</tr>
<tr>
<td>Pb-2(c)</td>
<td>6.20/6.20</td>
<td>6.20/6.20</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>75/75</td>
<td></td>
</tr>
<tr>
<td>Pb-1578(c)</td>
<td>3.10/3.10</td>
<td>3.10/6.20</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>75/75</td>
<td></td>
</tr>
<tr>
<td>Pb-ED01(c)</td>
<td>6.20/6.20</td>
<td>6.20/6.20</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>75/75</td>
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<tr>
<td>Pb-11(c)</td>
<td>6.20/6.20</td>
<td>6.20/6.20</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>75/75</td>
<td></td>
</tr>
<tr>
<td>Pb-8(c)</td>
<td>6.20/6.20</td>
<td>6.20/6.20</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>≥200/≥200</td>
<td>75/75</td>
<td></td>
</tr>
</tbody>
</table>

\(c\)Concentration in µg.mL\(^{-1}\) sufficient to inhibit 50% of the population of cells (IC50). Amphotericin B used as control; \(a\)Concentration in µg.mL\(^{-1}\) sufficient to inhibit 80% of the population of cells against Leishmania donovani (da Silva Filho et al., 2009). Sulfamethoxazol-trimetoprim used as control; \(b\)Concentration in µg.mL\(^{-1}\) sufficient to inhibit 94% of growth of UACC strain; \(c\)Concentration in µg.mL\(^{-1}\) sufficient to inhibit 99% of growth of MCF-7 strain; \(d\)Concentration in µg.mL\(^{-1}\) sufficient to inhibit 66% of the populations of cells.

significant cytotoxicity against several tumor cell lines as HL-60, K562, M4Beu, and HSC-2, with IC50 values ranging from 5.0 to 29.0 µg.mL\(^{-1}\) (Vechia et al., 2009). It is interesting to note that this triterpenoid exhibited IC50 values of 7.0 µg.mL\(^{-1}\) and 21 µg.mL\(^{-1}\) against Leishmania donovani (da Silva Filho et al., 2009) and Leishmania major (Takahashi et al., 2004), respectively. In our results it demonstrated IC50 value of 9.34 µg.mL\(^{-1}\) against *L. (L.) amazonensis*.

**Conclusions**

It was isolated five compounds where the chalcone 2 seems to be a new one. Although the other four compounds are usual, it was the first time that they were isolated in this genus and species. The chalcones 1 and 2 showed be active against all biological models researched moreover it was the first time that these compounds (1 and 2) were tested on these biological assays. Thus, these two chalcones are promising structures to the development of new drugs, showing the pharmacological potential of the *Blepharocalyx salicifolius*, a species little studied.

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Bioactivity of the compounds isolated from *Blepharocalyx salicifolius*
Ezequias P. Siqueira et al.


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Bioactivity of the compounds isolated from *Blepharocalyx salicifolius*
Ezequias P. Siqueira et al.

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