Maytenus salicifolia Reissek (Celastraceae): Evaluation of the Activity of Extracts and Constituents against Helicobacter pylori and Oral Pathogenic Microorganisms

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Abstract

Maytenus salicifolia Reissek is known as "cafezinho" in Minas Gerais State, Brazil. Its leaves have been used in folk medicine to treat stomach ulcers. The present study reports the isolation of 4'-O-methylepigallocatechin (1) and proanthocyanidin A (2) of root (1; 4.36%; 2; 2.17%) and branch (1; 4.16%; 2; 0.26%) polar extracts from M. salicifolia. These compounds were found in the roots of this species for the first time. These constituents were found in large amounts and it makes M. salicifolia a suitable source of these compounds. Moreover, the antimicrobial activity of polar (ethanolic from roots, ethanolic and ethylacetate from branches) and non-polar (hexanic from roots) extracts from this plant was evaluated in vitro against oral pathogens such as Candida albicans, Streptococcus mutans, Streptococcus sanguinis and Staphylococcus aureus. All extracts showed antimicrobial activity and C. albicans was the most sensitive microorganism. The triterpenes nepeticin, rigidolen, glochidone, 11-α-hydroxyglochidone and 16-β-hydroxypristimerina, which were previously isolated from this species, were also assayed against Helicobacter pylori. Rigidolen and 16-β-hydroxypristimerina exhibited activity against this bacterium. These results contribute to confirm the traditional use of M. salicifolia to treat ulcer and other gastrointestinal problems. They also indicated that the extracts from this plant have the potential to be used in the treatment of infectious diseases of the oral cavity.

Keywords: M. salicifolia; Celastraceae; antimicrobial properties; Helicobacter pylori; oral pathogens.
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1. Introduction

Oral cavity and gastrointestinal diseases are among the main health problems worldwide, many of them are caused by bacteria and fungi. The yeast Candida albicans and the lactic acid bacterium...
Bacterial infections are a significant cause of human diseases, with oral streptococci being the most common microorganisms found in the oral cavity. S. mutans is commonly identified in denture-related stomatitis and other forms of oral candidosis, and S. mutans is frequently associated with dental biofilm alterations and is considered one of the most important microorganisms involved in dental caries formation. Another bacterium of this genus, S. sanguinis, contributes for the action of other microorganisms that colonize the tooth surface, forming dental plaque and facilitating the development of caries and periodontal diseases. Another oral bacterium, Staphylococcus aureus, is related to acute dental infections, such as apical abscesses, jaw osteomyelitis and surgical complications after dental implant procedures. The high incidence of dental diseases is associated to other factors including the increased resistance of bacteria and fungi to the usual drugs. Thus, it is necessary to search new, safe, economic and effective options of new compounds to prevent and treat oral cavity diseases, and natural products, both plant extracts and isolated compounds, represent a good alternative.

Another microorganism of concern to human health is the Gram-negative bacterium Helicobacter pylori, which colonizes the hosts’ stomach for their lifetime. It has been acknowledged in the last two decades that this microorganism infects more than half of the world’s human population. H. pylori has been associated to peptic ulcer, gastrroduodenal diseases and cancer development, such as gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. In 1994, the World Health Organization classified H. pylori as a class I carcinogen for human gastric cancer, and since practically all infected people presented gastritis, it is expected that this bacterium plays a causative role early in the disease progression to adenocarcinoma.

Conventional treatments of infections caused by H. pylori are carried out based on the association of drugs, such as clarithromycin, amoxicillin, furazolidone, tetracycline and metronidazole, with bismuth or a proton pump inhibitor. However, the increase in bacterial resistance to antibiotics and the high cost of combined therapy hamper the adoption of this type of treatment in large scale.

In the context of ethnopharmacology, there are many reports about plants used to treat gastrointestinal disorders, especially about species belonging to Maytenus (M.) genus. M. aquifolium and M. rigida, for instance, presented properties against gastric ulcer. The medicinal use of M. ilicifolia, known in Brazil as ‘espinheira-santa’, has been reported since the 1920s. Pharmacological and clinic studies have been carried out since 1988 using extracts and constituents isolated from M. ilicifolia. The results are in accordance with scientific and popular experiments and contribute to the efficacy and therapeutic safety of the treatment of this disease. M. ilicifolia represents one phytotherapeutic whose pharmacological effects were recognized and established by Central de Medicamentos (CEME) of Ministério da Saúde, Brazil, and its use is totally safe.

M. genus is considered the largest group in the Celastraceae family. Up to now, there are about 80 recognized species included in this genus, which are distributed all over the Brazilian territory. Many biological properties popularly attributed to species of the M. genus were also experimentally established, such as antimitotic and antioxidant activity.

In the state of Minas Gerais, southeastern of Brazil, the leaves of M. salicifolia Reissek (Figure 1), commonly known as “cafezinho”, have been used in folk medicine to treat stomach ulcers. Decoction of its fresh leaves is popularly used to alleviate itches and skin allergies symptoms. From branches of M. salicifolia was previously reported the isolation of the pentacyclic triterpenes, 30-hydroxyfriedel-an-3-one, 3,16-dioxofriedelan, friedelin, lupin, betulin and lup-20(29)-en-3,30-diol and from the roots bark, the chalcone-diterpene adduct salicassin, and a quinone methide...
triterpenoid 16β-hydroxypristimerin.\textsuperscript{21} Pentacyclic triterpenes were also found in \textit{Austroplenckia populnea},\textsuperscript{22} \textit{M. gonoclada},\textsuperscript{23} \textit{M. robusta}\textsuperscript{24} and other species of the Celastraceae family of natural occurrence in Minas Gerais, Brazil.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Photos of \textit{M. salicifolia} Reissek, that is commonly found in Serra de Ouro Branco, MG, Brazil. A: Tree, B: leaves, C: seeds and D: part of the root.}
\end{figure}

Continuing our work on the chemical constituents of \textit{M. salicifolia}, the isolation of 4′-O-methylepigallocatechin (1) and proanthocyanidin A (2), compounds that were also isolated from other species of the Celastraceae family,\textsuperscript{23,25} is presently reported. The structural elucidation of these compounds was carried out using \textsuperscript{1}H and \textsuperscript{13}C NMR techniques, whose chemical shift assignments were very compatible with spectral data available in the literature.\textsuperscript{26}

Based on the use of this species in Brazilian traditional medicine, extracts and constituents isolated from \textit{M. salicifolia} were subjected to \textit{in vitro} assays to verify its effect on oral pathogens \textit{C. albicans}, \textit{S. mutans}, \textit{S. sanguinis} and \textit{S. aureus} that are fungi and bacteria commonly found in the oral cavity, and \textit{H. pylori}, which is associated to ulcer and other gastrointestinal disorders.

\section*{2. Materials and Methods}

\subsection*{2.1. General experimental procedures}
1H and 13C NMR spectra were run on a Bruker Avance DRX 400 spectrometer. The samples were dissolved in CDCl3 and TMS was used as internal standard. IR (film) spectra were recorded on a Bruker IFS 55 spectrophotometer, and UV spectra were performed on a Jasco V-560 spectrophotometer, for a sample dissolved in absolute EtOH. The column chromatography (CC) processes were carried out using silica gel 60 (Merck, 70-230 Mesh) and Sephadex LH-20 (Pharmacia). For the TLC analyses Sil 20 UV254, Panreac plates were used.

2.2. Plant material

*M. salicifolia* Reissek (Celastraceae) was collected from ‘Serra de Ouro Branco’, a mountain located in the Ouro Branco City region, MG, Brazil. The plant was identified by Drª. Rita Maria Carvalho-Okano, botanist of the Universidade Federal de Viçosa, MG, Brazil. A voucher specimen was deposited at the Herbarium José Badini of the Universidade Federal de Ouro Preto, MG, Brazil (Collection Nº. OUPR-18094). After collection, each part of the plant was separated and dried over kraft paper at room temperature (r.t.).

2.3. Extraction and compound isolation

The root bark of *M. salicifolia* (678.0 g) was subjected, at r.t., to exhaustive extraction with n-hexane, chloroform, ethyl acetate and finally with ethanol. After solvents removal at reduced pressure, the hexanic (HRE), chloroformic (CRE), ethyl acetate (ARE) and ethanolic (ERE) extracts were obtained. An aliquot (2.78 g) obtained from ethyl acetate extract (ARE, 9.6 g) was chromatographed on Sephadex LH-20 CC, eluted with a mixture of dichloromethane-acetone (3:2), and 26 fractions of 125 mL each were collected. Based on the similar profile observed by means of TCL, three groups of fractions were obtained. Due to its complexity, verified through TCL and the small amount (<15 mg) group 1 was discharged. From group 2 (208.5 mg) proanthocyanidin A (2) was isolated, which corresponds to 7.5 % of the aliquot. Group 3 was characterized as a red dark amorphous solid (417.0 mg), identified as 4’-O-methylepigallocatechin (1), corresponding to 15 % of the aliquot. In relation to ARE, compound 1 represented to 4.34% and 2 corresponded to 2.17%.

The compounds 3 to 7 were obtained from chloroform extract of root (CRE) bark of *M. salicifolia* as described in our previous work.21

The ethanolic extract from root bark (ERE; 86.4 g) of *M. salicifolia* was obtained as a brown amorphous solid. An aliquot of this solid material (300 mg) was subjected to silica gel CC, eluted with CH2Cl2-AcOEt in mixtures of increasing polarity, furnishing 47 fractions of 10 mL. Based on the TLC profile, fractions 21-29 were combined and gave compound 1 (20.0 mg), corresponding to 6.7 % of this aliquot (0.023% of ERE).

The branches of *M. salicifolia* (678.0 g) were also subjected, at r.t., to exhaustive extraction with n-hexane, CHCl3, ethyl acetate and finally with ethanol. The ethyl acetate extract obtained (ABE; 54.8 g) was fractioned through silica-gel 60 CC, using CH2Cl2-AcOEt as eluent in mixtures of increasing polarity, furnishing nineteen fractions of 500 mL each. Based on the similar profile observed through TLC, 7 groups of fractions were obtained. Group 4 gave 1 (2.28 g, 4.16% of this extract). From group 6 (23.59 g), a portion of 7.79 g was separated which was subjected to sephadex LH-20 CC, eluted to CHCl3-MeOH (1:1). Forty-two fractions were obtained. Based on the TLC profile, fractions 26-30 (1.72 g) were combined. A part of this group (255 mg) was submitted to silica-gel 60 flash CC using CH2Cl2-AcOEt as eluent in mixtures of increasing polarity, furnishing 40 fractions, which were grouped according to TLC profile. Group 1 gave rise to additional amount of compound 1 (23.0 mg), corresponding to 9.02 % of this portion. Group 3 was characterized as compound 2 (20.0 mg, 7.84
% of this aliquot, 0.26% of ABE). From the ethanolic extract (EBE), no compounds with suitable purity for NMR analysis were isolated.

2.4. Anti oral pathogens activity

The polar and non-polar extracts from roots (ERE, HRE) and branches (ABE, EBE) of *M. salicifolia* were evaluated against the oral pathogens *Candida albicans* (ATCC 18804), *Streptococcus mutans* (ATCC 70069), *Streptococcus sanguinis* (ATCC 10557) and *Staphylococcus aureus* (ATCC 12692). Samples of HRE, ERE, ABE and EBE were diluted in ethanol to reach concentrations of 50, 100 and 200 mg/mL. The antimicrobial assays were carried out using the agar diffusion method. Sterile blank discs treated with 20.0 µL of nystatin 1.000.000UI (Nys) (Sigma®, USA) or chlorhexidine 0.12% (Chlor) (Sigma®, USA) were respectively used as growth inhibition positive control. Discs treated with ethanol 70%, or distilled water, were used as negative controls. The assays, in triplicate, were repeated three times, in different moments. After a 24-hour incubation period at 35 °C, the diameters (mm) of growth inhibition zone induced by the samples of *M. salicifolia* were measured and compared with the antimicrobial standards: Nys and Chlor. The microorganisms were considered sensitive when the diameter of the inhibition zone presented values not 3 mm shorter than the positive control; moderately sensitive when the inhibition zone was bigger than 2 mm, but over 3 mm shorter than the positive control, and resistant when the diameter of the inhibition zone was ≤ 2 mm. The results were expressed as the median (M) ± standard deviation (SD). Minimum inhibitory concentration (MIC) was determined as the lowest concentration of the extract that inhibited the growth of the microorganisms subjected to the in vitro assay using the agar dilution method.

2.5. Anti H. pylori activity

Compounds 3 to 7 (Figure 2), previously isolated from *M. salicifolia*, were subjected to in vitro assays against *H. pylori* (INCS 00380). The culture of *H. pylori* incubated for 72 hours in soyabean casein digest medium (tryptone soya broth) was standardized by means of spectrophotometer, to reach final concentration of 1.33 x 10^8 UFC/mL (4.0 x 10^6 UFC/well). The bioassays were carried out in 96 well microplates, for all compounds, in five folds.

The samples were added to the wells from a 500 µg/mL (50 µg/well and final concentration of 250 µg/mL) solution, in tryptone soya broth. After that, 70 µL of the bacterium inoculum was added. The plates were incubated at 37 °C for 48 and 72 h. Negative (tryptone soya broth) and positive (ampicillin) controls were tested simultaneously. The inhibition growth percentage was determined for spectrophotometric reading at 630 nm using a micro-ELISA (Infinite M200, Tecan). Visual qualitative reading of microbial growth was also carried out using methylene blue 0.2 mg/L solution.

3. Results and Discussion

The ethyl acetate and ethanol extracts obtained from root bark (ARE and ERE) and branches (ABE) of *M. salicifolia* were subjected repeatedly to silica gel chromatographic processes, yielding the known flavonoids 4'-O-methyllepigallocatechin (1) and proanthocyanidin A (2) (Figure 2). The ^1H and ^13C NMR chemical shift assignments attributed to 1 and 2 (Spectral data in Supplementary Material) were in accordance with spectral data previously published. For the first time, these compounds in the roots of this species have been reported. The amounts found for 1 (8.81 %) and 2 (2.43 %) were considered significant and contribute to...
the use of *M. salicifolia* as an alternative source of these compounds, which show antioxidant properties.\(^{30}\) Certainly, the presence of these constituents, together with other flavonoids in the *Maytenus* species represent a determinant role in antiulcer and/or inhibition of gastric acid secretion processes,\(^{25,31}\) and support the use of *M. salicifolia* in Brazilian traditional medicine for the treatment of stomach diseases.

Due to the antimicrobial properties attributed to pentacyclic triterpenes and quinonemethides\(^{32,33}\) the lupanes nepeticin (3), rigidenol (4), glochidone (5), 11α-hydroxyglochidone (6) and the quinone methide 16β-hydroxypristimerin (7) (Figure 2) previously isolated from *M. salicifolia*,\(^{21}\) were subjected to *in vitro* assays to evaluate its properties against the pathogenic gastrointestinal bacteria *H. pylori*. Compounds 4 and 7 showed potential for *H. pylori* inhibition, with 7 being the most active (Table 1). In the assay conditions no activity was detected for the other lupane triterpenes. The active compounds 4 and 7, associated to flavonoids 1, 2, and/or other constituents previously identified in *M. salicifolia*, such as pinostrobin and ferruginol,\(^{21}\) which display the same activity,\(^{34,35}\) support the popular use of this plant. In addition, *M. salicifolia* is used as an infusion, which should be mainly constituted by phenolic compounds that are correlated to popular treatments of gastrointestinal disorders.

![Figure 2. Chemical structure of compounds 1 to 7 isolated from *M. salicifolia*](image-url)
**Table 1.** In vitro inhibition of *H. pylori* growth induced by pentacyclic triterpenes and quinonemethides isolated from *M. salicifolia*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Growth inhibition (% M ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(250 µg/mL)</td>
<td>48 h</td>
</tr>
<tr>
<td>Nepeticin (3)</td>
<td>nd</td>
</tr>
<tr>
<td>Rigidenol (4)</td>
<td>26.0 ± 7.0</td>
</tr>
<tr>
<td>Glochidone (5)</td>
<td>nd</td>
</tr>
<tr>
<td>11α-Hydroxyglochidone (6)</td>
<td>nd</td>
</tr>
<tr>
<td>16β-Hydroxypristimerin (7)</td>
<td>84.0 ± 4.0</td>
</tr>
<tr>
<td>Ampicillin (Positive control)</td>
<td>100.0 ± 6.0</td>
</tr>
</tbody>
</table>

nd = not detected in the assay conditions

Mean and Standard Deviation (M ± SD) inhibition zones

The results of antimicrobial assays using extracts of *M. salicifolia* obtained from its branches treated with ethyl acetate (ABE) and from its roots treated with hexane (HRE) and with ethanol (ERE) against oral pathogenic microorganisms are presented in Table 2. All extracts of *M. salicifolia* presented properties that inhibit the growth of all microorganisms subjected to assays. However, when the efficacy of the samples was compared to positive controls, activity variations were noticed. In comparison to nystatine, all extracts were considered moderately active against *C. albicans*, because the diameter of the respective inhibition zones was much higher than 2 mm, but over 3 mm shorter than the control. When the inhibition of the growth of this yeast was compared to chlorhexidine (0.12%), the sample ERE in all concentrations evaluated and HRE (50 mg/mL) were more active than that one.

In relation to the bacteria subjected to in vitro assays, was observed that *S. mutans* was seen to be sensitive to HRE in all concentrations evaluated. Regarding all other samples, moderate inhibition of this bacterium growth was observed. *S. sanguinis* was the most resistant microorganism, being sensitive only to HRE at concentrations of 200 mg/mL. For all samples assayed, moderate activity was observed. The most pronounced inhibition of *S. aureus* growth was induced by ERE at concentration of 200 mg/mL and EBE at concentration of 50 mg/mL, and for HRE at concentrations ≥100 mg/mL. For ABE, good growth inhibition activity against *S. aureus* at concentrations of 50 mg/mL and 200 mg/mL was observed.
**Table 2.** Antimicrobial susceptibility of oral pathogenic microorganisms to *M. salicifolia* extracts. Mean and Standard Deviation (M ± SD) inhibition zones. Chlor = chlorhexidine and Nys = nystatine, used as positive control

<table>
<thead>
<tr>
<th>Extracts of <em>M. salicifolia</em> (mg/mL)</th>
<th>Diameter (mm) of inhibition growth zone (M ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. albicans</em></td>
</tr>
<tr>
<td>ERE*</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>17.6 ± 0.82</td>
</tr>
<tr>
<td>100</td>
<td>18.0 ± 0.00</td>
</tr>
<tr>
<td>200</td>
<td>18.0 ± 0.00</td>
</tr>
<tr>
<td>EBE**</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>16.3 ± 1.03</td>
</tr>
<tr>
<td>100</td>
<td>14.5 ± 0.13</td>
</tr>
<tr>
<td>200</td>
<td>14.0 ± 0.00</td>
</tr>
<tr>
<td>ABE***</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>12.0 ± 0.00</td>
</tr>
<tr>
<td>100</td>
<td>11.5 ± 0.13</td>
</tr>
<tr>
<td>200</td>
<td>10.5 ± 1.03</td>
</tr>
<tr>
<td>HRE****</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>19.3 ± 0.33</td>
</tr>
<tr>
<td>100</td>
<td>14.0 ± 0.00</td>
</tr>
<tr>
<td>200</td>
<td>13.0 ± 0.00</td>
</tr>
</tbody>
</table>

Chlor (0.12%)  14.5 ± 0.15  18.8 ± 0.14  17.6 ± 0.22  15.0 ± 0.00
Nys 1.000.000UI  22.9 ± 0.64  -  -  -
Ethanol 70%  0.00 ± 0.00  0.00 ± 0.00  0.00 ± 0.00  0.00 ± 0.00
Distilled water  0.00 ± 0.00  0.00 ± 0.00  0.00 ± 0.00  0.00 ± 0.00

*ERE*: ethanolic extract of roots  
**EBE**: ethanolic extract of branches  
***ABE**: ethylacetate extract of branches  
****HRE**: hexanic extract of roots

The results related to minimum inhibitory concentration (MIC) found for the microorganisms (Table 3), ranged from 10.0 to 44.0 mg/mL. The variation of MIC observed for *C. albicans* oscillated between 10.0 to 22.0 mg/mL, which was considered the most susceptible among the microorganisms treated with extracts of *M. salicifolia*. 

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*Magalhães, C. G. et al.*

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salicifolia. The most susceptible bacterial strain was the cariogenic pathogen S. mutans (MIC ranging from 11.0 to 40.0 mg/mL), followed by S. sanguinis (MIC varying between 20.0 to 44.0 mg/mL). S. aureus was the less sensitive bacterium to M. salicifolia extracts (MIC = 40.0 to 44.0 mg/mL). For the positive controls used, chlorexidine MIC was 0.02 mg/mL and nystatine MIC was 0.004 mg/mL.

**Table 3.** Minimum inhibitory concentration (MIC) of M. salicifolia extracts tested against oral pathogenic microorganisms by means of micro-dilution method. Chlor = chlorhexidine and Nys = nystatine, used as positive control.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>ERE*</th>
<th>EBE**</th>
<th>ABE***</th>
<th>HRE****</th>
<th>Chlor</th>
<th>Nys</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>22.0</td>
<td>-</td>
<td>0.004</td>
</tr>
<tr>
<td>S. aureus</td>
<td>40.0</td>
<td>40.4</td>
<td>40.0</td>
<td>44.0</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>S. mutans</td>
<td>11.0</td>
<td>40.2</td>
<td>40.0</td>
<td>11.0</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>S. sanguinis</td>
<td>44.0</td>
<td>40.2</td>
<td>20.0</td>
<td>44.0</td>
<td>0.02</td>
<td>-</td>
</tr>
</tbody>
</table>

*ERE: ethanolic extract of roots
**EBE: ethanolic extract of branches
***ABE: ethylacetate extract of branches
****HRE: hexanic extract of roots

The HRE activity can be associated with tingenone and lupeol, which were previously isolated from this hexanic extract and from other species belonging to family Celastraceae. Antimicrobial properties have been already attributed to tingenone and lupeol. The occurrence of 4′-O-methylepigallocatechin and proanthocyanidin A in polar extracts from M. salicifolia and from other species belonging to M. genus justifies the antimicrobial activity herein observed, which corroborated previously data reported for these compounds.

**4. Conclusions**

In this work is described for the first time the occurrence of 4′-O-methylepigallocatechin (1) and proanthocyanidin A (2) in the roots of M. salicifolia and the expressive activity of M. salicifolia extracts against oral pathogens. The active compounds rigidienol (4) and 16β-hydroxypristimerin (7) against H. pylori contributed to the scientific support for the popular use of this species in the treatment of ulcer and other gastrointestinal disorders. These results showed that M. salicifolia can be a promising basis for further investigation in the discovery of new natural antibiotic compounds.

**Acknowledgments**

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