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ANTIMICROBIAL ACTIVITY OF GUNNERA TINCTORIA (MOL.) MIRB. AGAINST PORPHYROMONAS GINGIVALIS IN PERIODONTAL DISEASE

Jorge Gálvez	Facultad de Medicina, Universidad Andres Bello, Campus República, Santiago
Maité Rodríguez-Díaz*	Facultad de Medicina, Universidad Andres Bello, Campus República, Santiago *Corresponding Author
Carolina Otero	Center for Integrative Medicine and Innovative Science, Facultad de Medicina, Universidad Andres Bello, Santiago, Chile
Fernando Torres	Facultad de Medicina, Universidad Andres Bello, Campus República, Santiago
Jose Manuel Delgado	Facultad de Medicina, Universidad Andres Bello, Campus República, Santiago
Denisse Bravo	Facultad de Odontologia, Universidad de Chile

ABSTRACT In Chile, in particular Mapuches, traditional medicine, the medicinal plant *Gunnera tinctoria* (Mol.) Mirb. is used to relieve sore throats and irritated gums.

Aim of the Study: To evaluate and quantify the antimicrobial activity of petiole extracts of *G. tinctoria* (Mol.) Mirb. against *Porphyromonas* gingivalis, the principial etiologic agent in periodontal disease.

Materials and Methods: A sequential extraction of macerated petioles of *G. tinctoria* (Mol.) Mirb. was carried out, and the antimicrobial activity of each extract was tested in a series of disk diffusion, turbidity and bacterial viability assays. MIC was determined for extracts showing a antimicrobial activity.

Results: Methanolic and ethanolic extracts showed antimicrobial activity against *P. gingivalis*, with a MIC of 2.0 mg/mL for both extracts. Use of the methanolic extract resulted in a smaller number of colony forming units in a quantitative bacterial viability assay.

Conclusions: This is the first study on the antimicrobial activity of *G. tinctoria* (Mol.) Mirb. in periodontal disease. In view of the accelerated spread of bacterial antibiotic resistance, the use of traditional medicinal plants represents an attractive alternative strategy with few adverse effects. We suggest that *G. tinctoria* (Mol.) Mirb. could be used as an additional tool in the periodontal disease treatment.

KEYWORDS : Gunnera tinctoria, antimicrobial activity, periodontal disease, Porphyromonas gingivalis, methanolic extracts

1. Introduction

Rise in sugar and tobacco consumption has led to a steep increase in chronic diseases on the oral cavity. Beyond their effect on the oral cavity itself, they may have important implications for general health: nutritional deficiencies, cardiovascular problems, pneumonia and diabetes are just a few examples of systemic diseases associated with oral pathologies (Lindhe et al., 2008). By affecting both physical and psychological health, oral pathologies therefore have a significant negative impact on patient quality of life (Hobdell et al., 2003; Sheiham, 2005).

Gingivitis and periodontitis are the most common oral pathologies (World Health Organization, 2012). Gingivitis is an inflammation which exclusively affects gums, and which is mediated by the accumulation of microorganisms (Wolf et al., 2004). In Chile, around 55% of children (Soto et al., 2007a) and 67% of adolescents (Soto et al., 2007b) are affected by gingivitis. When it is not treated in a timely fashion, inflammation can spread from gums to the rest of the dental support tissues; this is called periodontitis (US National Library of Medicine, 2015). Periodontitis causes gums inflammation, cementum, alveolar bone and periodontal ligament, potentially leading to a progressive loss of the alveolar bone and, as a consequence, tooth loosening and loss (Newman et al., 2015).

While gingival and periodontal diseases are multifactorial, their main cause is considered to lie in a disequilibrium of the microbiota forming the bacterial plaque lining the surface of the oral cavity (Hajishengallis, 2014). Periodontoal disease is associated with a microbiotic disequilibium in the subgingival plaque, which is locatal below the gingival sulcus and populated mainly by Gram negative bacteria with a proteolytic metabolism (Lindhe et al., 2008). Among these, the Gram negative coccobacillus *Porphyromonas gingivalis* is one of the principal etiological agents in periodontal disease (Byrne et al., 2009; Díaz Zúñiga et al., 2012; Farias Rodríguez et al., 2003; Ramos Perfecto et al., 2014). In recent studies, *P. gingivalis* has been characterized as a key pathogen and cornerstone for the development of periodontal disease, since, though not abundant among bacteria populating the plaque, it causes a disequilibrium of the microbiota, which ultimately leads to the increase of the pathogenic bacteria population (Hajishengallis, 2014; Hajishengallis and Lamont, 2014).

Alongside surgery and plaque control, main treatment for periodontal disease consists in the use of antibiotics, both systemic (such as metronidazole, ciprofloxacin and clindamycin) and local (such as doxycycline gel and tetracycline fibers) (Lindhe et al., 2008; Olate M. and Soto A., 2007; Slots, 2005). However, like other pathogenic bacteria, *P. gingivalis* is evolving resistance to different antibiotics (Sanai et al., 2002; Sweeney et al., 2004), and the biofilm formed by the bacterial plaque offers additional protection to the pathogen (Stewart, 2002). The search of novel pharmacological therapies is therefore relevant for the control of periodontal disease.

Since their earliest origins, humans have used plant species to preserve their health, and this traditional use is increasingly influence modern medicine and dentistry. In Chile, species *Gunnera tinctoria* (Molina) Mirbel (Gunneraceae), locally known as “Nalca” or “Pangue”, has traditionally been used to treat a wide range of conditions, including hemorrhages, abundant and painful periods, dysentery, sore throats and gums irritations. The latter use may suggest that this species could be of use in the treatment and prevention of periodontal disease (MINSAL, 2009). Further traditional uses outside medicine include consumption of its thick and fleshy petioles, the use of its roots to produce a black dye, and the use of its leaves in Chiloé cuisine (Valdebenito et al., 2003). Genus *Gunnera* comprises 14 species native to the Southern Hemisphere, which are distributed across large parts of South America, including Chile, Argentina, Peru, Ecuador, Colombia and Venezuela (Belov, 2009; Muñoz-Schick, 1980; Williams et al., 2005).

Several studies have found antimicrobial activity against Gram negative bacteria, including *Escherichia coli*, in a different species of the genus, *G. perpensa* (Drewes et al., 2005; McGaw et al., 2005; Nkomo and Kambizi, 2009). For *Gunnera tinctoria* (Mol.) Mirb., antimicrobial activity against *Micrococcus luteus* and *Helicobacter pylori* has been found in extracts of its leaves and petioles (Barreto et al., 2006; Hebel et al., 2013). This is consistent with the finding of

triterpenes in this species (Muñoz et al., 2004). Based on this, it may be hypothesized that *Gunnera tinctoria* (Mol.) Mirb. present antimicrobial activity against *P. gingivalis*, potentially representing an alternative treatment for periodontal diseases.

Here, we investigate for the first time the use of *Gunnera tinctoria* (Mol.) Mirb. in periodontal disease. Then, our study represents the first step towards an integration of traditional medicinal plant and modern medicine. Here, we evaluated the antimicrobial activity against *P. gingivalis* of a series of extracts obtained from the petioles of *Gunnera tinctoria* (Mol.) Mirb. To achieve this, we carried out a sequential extraction from macerated petioles and evaluated the antimicrobial potential of each extract by performing disk diffusion, turbidity and bacterial viability assays.

2. Materials and methods

2.1 Collection and preparation of plant material

Petioles of *Gunnera tinctoria* (Mol.) Mirb. were collected in April 2014 in the Araucania Region of Chile by Agro Santa Fe Ltda. Petioles were washed with cold water and left to dry over five weeks at room temperature, followed by 48 hours at 35°C in drying stove. Petioles were knife grounded (Retsch, SM300 model) and sieved up to a particle size of 500 µm. The voucher material was deposited at Herbarium of Pharmacy School, Universidad de Chile. Dry and fractionated plant material was stored in a dry environment at room temperature.

2.2 Sequential extraction

Sequential extraction entails an extraction in several cycles using solvents of increasing polarity. Here, we used hexane, dichloromethane, ethyl acetate, methanol, ethanol, and water. We followed the protocol of Villar del Fresno (1999). Briefly, 100 g dried and ground plant material were left to stand (macerate) in 1 L solvent for 48 hours. Following this, the extract was separated from the remaining plant material by filtration, and the plant material was left to dry at room temperature in a tray covered with pierced aluminum foil to allow the entry of air. All steps were then repeated with the remaining plant material and the following, more polar, solvent. All extracts were dried in a rotary evaporator until complete evaporation of the solvent and stored in amber bottles at 4° C.

2.3 *P. gingivalis* culture

P. gingivalis strain ATCC 33277 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and kept at -80°C. In preparation for the antimicrobial assays, the strain was cultivated in hemin-menadione (5 µg/mL) enriched BHI medium (MDM Cientifica, Medellín, Colombia) for seven days at 37 °C, under anaerobic conditions. Anaerobic conditions were created using the AnaeroGen™ system (ThermoFisher).

2.4 Microbiological tests

Disk diffusion and dilution assyas were carried out according to Díaz et al. (2015).

2.4.1 Disk diffusion assay

Hemin-menadione enriched BHI medium was inoculated with *P. gingivalis* and adjusted to McFarland standard 3 and an OD_{600} of 0.3. 100 µL of this broth were plated onto hemin-menadione blood agar (Becton Dickinson, Heidelberg, Germany) using the lawn plate method. For disk diffusion assay, 5 mm filter paper disks were infused with 10 µL of each extract dissolved in its original solvent at different concentrations. From a 100 mg/mL stock solution of each extract, a serial dilution was prepared to obtain concentrations of 10 mg/mL, 5 mg/mL and 2.5 mg/mL. For each extract, a disk infused with 10 µL of the respective solvent was used as a control. The plates were left to incubate for seven days under unaerobic conditions, at a temperature of 37°C. Following this, diameter of the inhibition zone around the disk was measured.

2.4.2 Turbidity tests for bacterial growth

Antimicrobial activity of *G. tinctoria* extracts in liquid medium was investigated in three approaches. As part of each approach, those extracts not presenting any antimicrobial activity were excluded from further analysis.

The first approach consisted in a turbidity test of each extract re-dissolved in its original solvent. This was followed by a turbidity test of each extract re-dissolved in water. The results of this second test were confirmed in a bacterial viability assay on agar plates. Finally, those extracts that had shown antimicrobial activity in the two previous tests, were subjected to a further liquid medium growth test based on optical density measurements. This last test again used the aqueous solutions of all extracts, and results were again confirmed in a bacterial viability test on agar plates.

All approaches tested a range of dilutions of the re-dissolved extracts. In the last approach, minimum inhibitory concentrations were determined for those extracts showing antimicrobial activity.

For the first turbidity test, all six extracts were re-dissolved in their original solvents (hexane, dichloromethane, ethyl acetate, methanol, ethanol and water), achieving final concentrations of 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL and 0.625 mg/mL for each extract. Pure solvent was used as a control for each extract. These solutions were mixed with 500 µL of *P. gingivalis*-inoculated broth (OD_{600} = 0.3) and left to incubate for five days under anaerobic conditions at 37°C. At the end of this period, turbidity was assessed to determine bacterial growth.

For the second turbidity test, aqueous, methanolic and ethanolic extracts were each re-dissolved in autoclaved distilled water. Final concentrations for the methanolic and ethanolic extracts were 5.0 mg/mL, 4.0 mg/mL, 3.0 mg/mL and 2.4 mg/mL; for the aqueous extract, final concentrations were 5.0 mg/mL, 4.5 mg/mL, 4.0 mg/mL, 3.5 mg/mL, 3.0 mg/mL and 2.5 mg/mL. These dilutions were mixed with 500 µL of the *P. gingivalis*-inoculated broth (OD_{600}= 0.3). For two controls tubes, only water was added instead of extract. Inoculated solutions were left to incubate for five days under anaerobic conditions at 37°C. At the end of this period, bacterial growth was determined by turbidity. For each extract and each dilution, bacterial viability was determined on hemin-menadione blood agar plates. Plates were incubated for seven days at 37°C. At the end of this period, plates were checked for the presence of *P. gingivalis* colonies.

For the third and final liquid medium growth test, ehanolic and methanolic extracts were re-dissolved in autoclaved distilled water to achieve final concentrations of 2.0 mg/mL, 1.5 mg/mL, 1.0 mg/mL and 0.5 mg/mL. These solutions were mixed with 500 µL of *P. gingivalis*-inoculated broth (OD_{600}= 0.3) and left to incubate for five days at 37°C under anaerobic conditions. At the end of this period, OD_{600} of each solution was measured in an UV/vis spectrophotometer (Jenway, modelo 6405). Measurements were performed in triplicates. In order to confirm the inhibition of *P. gingivalis* growth, a bacterial viability test was conducted on hemin-menadione blood agar plates. For each dilution of each extract, a plate was inoculated with 100 µL of a 1:100000 dilution of the original dilution. Plates were incubated for seven days under anaerobic conditions at 37°C and screened for the presence of *P. gingivalis* colonies, which were counted under stereoscopic magnifier (C. Zeiss, Germany). The program GraphPad Prism 6 was used for statistical evaluation of differences in OD and colony numbers between different extracts and dilutions.

3. Results

3.1 Extraction from *Gunnera tinctoria* (Mol.) Mirb.

Mass extracted from 100.013 g plant material ranged from 0.3996 g for the hexane extract, to 8.3694 g for the methanolic extract.

3.2 Disk diffusion test

Results of the disk diffusion test are summarized in Table 1. Ethyl acetate, ethanolic and methanolic extracts resulted in an inhibition zone around the disk in all their concentrations. The inhibition zone with the greatest diameter was obtained from the ethanolic extract at a concentration of 2.5 mg/mL. Hexane and dichloromethane extracts did not show inhibition of bacterial growth at any of the concentrations tested.

Table 1. Diameter of the inhibition zones obtained for different concentrations of the different extracts of *Gunnera tinctoria* (Mol.) Mirb. on a *P. gingivalis* lawn. A dash indicates the absence of bacterial growth inhibition.

Concentration (mg/mL)	Hexane (mm)	Dichloromethane (mm)	Ethyl acetate (mm)	Methanol (mm)	Ethanol (mm)	Water (mm)
10.0	—	—	6	9	9	—
5.0	—	—	6	7	8	7
2.5	—	—	8	9	10	—
solvent	—	—	7	12	8	—

3.3 Turbidity test for bacterial growth

The first liquid medium growth test evaluated the turbidity of solutions of each extract re-dissolved in its original solvent and inoculated with *P. gingivalis*. Methanolic, ethanolic and aqueous extracts inhibited bacterial growth, all at a 5 mg/ml concentration. Solutions containing the extracts obtained from hexane, dichloromethane and ethyl acetate were turbid after incubation, showing bacterial growth and therefore indicating that none of the three extracts contained an antimicrobial agent active against *P. gingivalis*. Based on this, and backed by the results of the disk diffusion test, only methanolic, ethanolic and aqueous extracts were investigated further.

Methanolic, ethanolic and aqueous extracts were re-dissolved in water for the second liquid medium growth test. In addition to a visual turbidity inspection, a bacterial viability test was carried out for all dilutions. The solutions containing the aqueous extract at all dilutions were found to be turbid after the incubation period, and bacterial growth was confirmed for all dilutions by the bacterial viability assay. The aqueous extract therefore did not show any antimicrobial propierties against *P. gingivalis.*

None of the dilutions of the ethanolic and methanolic extracts were turbid after the incubation, and the bacterial viability assays were negative for all tested dilutions of both extracts. It was therefore concluded that ethanolic and methanolic extracts both showed antimicrobial activity against *P. gingivalis*. This was confirmed by the negative results, which were both turbid after incubation, indicating that the inhibition of bacterial growth was indeed due to the presence of the respective extracts.

3.4 Optic density measurements

OD measurements were taken in ethanolic and methanolic extracts re-dissolved in water after incubation with *P. gingivalis*. For each extract, concentrations of 2.0 mg/mL, 1.5 mg/mL, 1.0 mg/mL and 0.5 mg/mL were tested. Results are shown in Figure 1. In line with expectations, the higher the concentration of each extract, the lower the DO_{600}. At 2.0 mg/mL, the OD_{600} is equal to zero for both extracts, indicating inhibition of bacterial growth by both extracts at this concentration. Differences in OD_{600} between equivalent concentrations of both extracts were not statistically significant ($P > 0.05$).

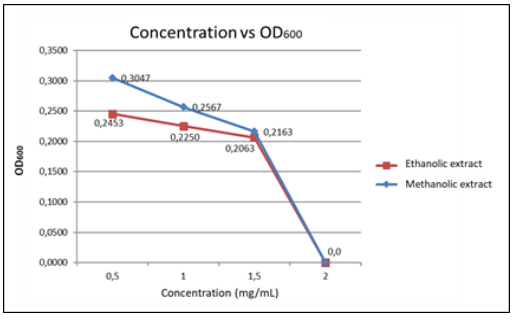

Figure 1. Optical density of *P. gingivalis* suspensiuons cultivated with different concentrations of methanolic and ethanolic extracts of *Gunnera tinctoria* (Mol.) Mirb. The average of three measurements is shown.

In addition to optical density measurements, a bacterial viability assay was carried out for each dilution of each extract. Results are summarized in Figure 2.

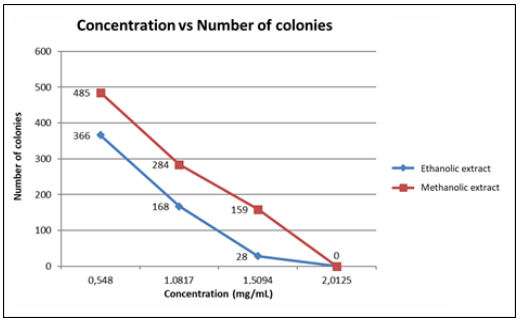

Figure 2. Number of colonies in a bacterial viability assay of *P. gingivalis* suspensions cultivated with different concentrations of methanolic and ethanolic extracts of *Gunnera tinctoria* (Mol.) Mirb. Average from three plates is shown.

In line with the results of the turbidity test and OD measurements, no colonies were observed at 2.0mg/mL for both extracts. This indicates that minimum inhibitory concentration (MIC) for both extracts is 2.0 mg/mL. (Table 2)

Table 2. Minimum inhibitory concentration (MIC) (mg/mL) of the methanolic and ethanolic extracts of *Gunnera tinctoria* (Mol.) Mirb.

Minimum inhibitory concentration (MIC) (mg/mL)	OD_{600} Media Methanolic extract	OD_{600} Media Ethanolic extract
2,0125	0,003 ±0,006	0,000 ±0,000

At all other concentrations, the number of colony forming units was larger for ethanolic than for methanolic extract ($P < 0.05$ for all comparisons). This suggests that the methanolic extract has a greater antimicrobial activity than the ethanolic extract, and hence the greatest antimicrobial activity against *P. gingivalis* off all extracts tested here.

Discussion

In this study, we carried out a series of bacterial growth and viability assays in order to determine the antimicrobial activity of petiole extracts of *Gunnera tinctoria* (Mol.) Mirb., a plant which has traditionally been used to treat gums irritations and sore throats (MINSAL, 2009). In this study, we found that the methanolic and ethanolic extracts exhibited a clear antimicrobial activity against *P. gingivalis*.

Extraction with water, ethanol and methanol were most efficient (data not shown), indicating that most extracted compounds exhibit a polar nature. We carried out a series of growth inhibition assays, including disk diffusion and turbidity tests. These assay did not find any antimicrobial activity of hexane, dichloromethane, ethyl acetate and, finally, aqueous extracts against *P. gingivalis*; they were therefore excluded from further testing. Both ethanolic and methanolic extracts exhibited antimicrobial activity against *P. gingivalis* in all assays. Based on optical density measurements and bacterial viability assays on agar, MIC of both extracts was determined to be 2.0 mg/mL, since at that concentration, bacterial growth was shown to be completely inhibited.

This is the first study on the activity of *Gunnera tinctoria* (Mol.) Mirb against *P. gingivalis*, and therefore the obtained MIC can only be compared to studies of other species of *Gunnera*, or to *Gunnera tinctoria* (Mol.) Mirb. activity against other bacterial species. Studies on *Gunnera perpensa* have found MIC values ranging from 0.5 mg/mL to 1.0 mg/mL against different Gram negative bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Shigella flexneri* and *Serratia marcescens* (Nkomo and Kambizi, 2009). In a different study a MIC of 6.25 mg/mL has been reported against *Escherichia coli* (McGaw et al., 2005). For *Gunnera tinctoria* (Mol.) Mirb., a MIC between 0.128 mg/mL and 0.512 mg/mL has been reported against *Escherichia coli* (Hebel et al., 2013).

Although, the MIC was found to be the same for both extracts, bacterial viability assay revealed statistically significant differences in the number of colony forming units between both extracts. Specifically, the methanolic extract was found to result in a smaller number of colonies. A possible explanation lies in the sequence of extraction: because the methanolic extract was obtained before the ethanolic one, most compounds of interest may have been extracted into methanol, leaving a smaller fraction for extraction into ethanol. Our results are in line with previous studies, which have used the same extraction protocol and have also found a greater biological activity and efficiency of methanolic compared to ethanolic extracts (Hebel et al., 2013). They are also consistent with the fact that practically all compounds of phytochemical interest have a similar solubility in methanol and ethanol (Alvarez and Bague, 2012). Iuak et al., 2003 reports antimicrobial activity of many methanol plant extracts against periodontophatic bacteria, finding that the alcoholic extracts presented smaller MIC than the decocts against *P. gingivalis* (Iuak et al., 2003).

Optical density measurements did not reveal any statistically significant differences between the two extracts; this could be explained by the fact that OD measurements do not indicate bacterial

viability. Solutions of both extracts may therefore have contained a similar number of bacteria at each concentration; however, their viability may well have been different, as shown after culturing on agar plates.

The antimicrobial activity of ethanolic and methanolic extracts of *Gunnera tinctoria* (Mirb.) Mol. is mainly due to the presence of polyphenolic compounds, including flavonoids, tannins and terpenes (Ojeda, 2013). These compounds exhibit antioxidant, astringent, analgesic and anti-inflammatory properties (Escamilla et al., 2009; López et al., 2012; Martínes-Flórez et al., 2002). In particular, such properties have been described for the flavonoids quercetin and kaempferol (Calderón-Montaño et al., 2011; Pastene et al., 2012). In line with our findings, a series of studies on *Gunnera tinctoria* (Mol.) Mirb. have found that extracts show anti-inflammatory, antioxidant, analgesic and wound healing properties (Hebel et al., 2013). Also

In the context of periodontal disease, in addition to combating bacterial population underlying this disease, these extracts may hence mitigate inflammation and promote tissues healing affected by periodontal damage or surgical treatment, as well as act as a local analgesic, thereby supporting an entire recovery from the disease.

The present study represents a first step in the development of new strategies for the periodontal diseases management associated with bacterial infections. Nevertheless, many bacterial species contribute to periodontal disease, and many metabolic interactions between bacteria of the subgingival plaque play an important role in their survival in the periodontium (Newman et al., 2015). It is therefore, desirable to confirm its antimicrobial activity of *Gunnera tinctoria* (Mol.) Mirb. extracts on other pathogens associated with periodontitis, such as *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans* and *Treponema denticola*. The more bacterial pathogens are affected by *Gunnera tinctoria* (Mol.) Mirb. extracts, the greater possible effect on the disease. Likewise, it would be necessary to test the effect of *Gunnera tinctoria* (Mol.) Mirb. on the normal oral microflora. This is particularly important because an imbalance in the microbiota, be it caused by *P. gingivalis* or by other factors, may trigger the development of periodontal disease, as discussed above. Finally, toxicity tests should be carried out on the cells of the gingival epithelial before *Gunnera tinctoria* (Mol.) Mirb. can be considered as a viable alternative treatment.

In conclusion, we found that methanolic and ethanolic extracts of *Gunnera tinctoria* (Mol.) Mirb. exhibit antimicrobial activity against *P. gingivalis* in vitro, with a MIC of 2.0 mg/mL for both extracts. Our findings represent a promising first step towards the development of an alternative treatment strategy in periodontal disease based on traditional medicinal plant.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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