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Chemistry

Antibacterial screening of hexane extracts from *Psidium myrtoides*, a Brazilian native plant

Triagem antibacteriana de extratos hexânicos de *Psidium myrtoides*: uma planta nativa brasileira

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ABSTRACT

Even though Brazil has a large biodiversity of medicinal plants, many of them have not had their chemical and biological potential acknowledged. Several medicinal plants are rich in secondary metabolites with broad antibacterial activity, a relevant fact since many pathogenic bacteria are resistant to conventional antibiotics. *Psidium myrtoides*, a species of plant in the Myrtaceae family that is known for its sweet purple fruit, should be highlighted in this field. It is a medicinal plant with strong antibacterial, antifungal, anti-inflammatory, antioxidant, antitumoral, and herbicidal potential. This study aimed at investigating, for the first time, the antibacterial activity of hexane extracts from *P. myrtoides* dry leaves – which were collected in both dry and rainy seasons – and the dry pericarp of unripe fruit. Their antibacterial activity was evaluated *in vitro* by the broth microdilution method on 96-well plates, and their Minimum Inhibitory Concentration (MIC) values were expressed as µg/mL. The following bacteria were assayed: *Streptococcus mutans, S. sobrinus, S. mitis, S. salivarius, S. sanguinis, Enterococcus faecalis, Lactobacillus casei, Xanthomonas citri* (resistant, tolerant, and sensitive to copper), *Mycobacterium tuberculosis, M. avium, and M. kansasii*. The resulting MIC values of all extracts under study were promising since they ranged from 31.25 to 500 µg/mL. This study revealed the *in vitro* potential of *P. myrtoides* against different bacterial strains and reinforced the need for further phytochemical investigations that aim at isolating its bioactive chemical constituents.

Keywords: Natural antimicrobials; Hexane extracts; Brazilian cerrado

RESUMO

O Brasil apresenta grande biodiversidade de plantas medicinais, muitas delas com seus potenciais químico e biológico ainda desconhecidos. Muitas plantas medicinais são ricas em metabólitos secundários com ampla ação antibacteriana, fato relevante tendo em vista que muitas bactérias patogênicas são resistentes aos antibióticos convencionais. Nesse cenário, destaca-se a espécie *Psidium myrtoides*, uma Myrtaceae conhecida pelos seus frutos roxos de sabor adocicado. É uma planta medicinal com fortes potenciais antibacteriano, antifúngico, anti-inflamatório, antioxidante, antitumoral e herbicida. O presente estudo visou investigar pela primeira vez a atividade antibacteriana dos extratos hexânicos preparados a partir das folhas secas de *P. myrtoides* coletadas em duas épocas do ano (seca e chuvosa) e também do extrato hexânico do pericarpo seco dos frutos verdes. A atividade antibacteriana foi avaliada *in vitro* pelo método de microdiluição em caldo utilizando microplaca de 96 poços e os valores de concentração inibitória mínima expressos em µg/mL (CIM). Várias bactérias foram testadas, sendo elas: *Streptococcus mutans, S. sobrinus, S. mitis, S. salivarius, S. sanguinis, Enterococcus faecalis, Lactobacillus casei, Xanthomonas citri* (resistente, tolerante e sensível ao cobre), *Mycobacterium tuberculosis, M. avium e M. kansasii*. Os valores de CIM encontrados foram promissores para todos os extratos testados estando entre 31,25 e 500 µg/mL. O presente estudo revelou o potencial *in vitro* de *P. myrtoides* contra diferentes cepas bacterianas e reforçou a necessidade de futuras investigações fitoquímicas visando o isolamento de seus constituintes químicos bioativos.

Palavras-chave: Antimicrobianos naturais; Extratos hexânicos; Cerrado brasileiro

1 INTRODUCTION

Ethnomedicine and phytotherapy have been widely practiced all over the world. Several medicinal plants have been used by different peoples but few have had their activities scientifically confirmed. However, knowledge and consolidated popular use have been used as guidelines for chemical and pharmacological research (Oliveira et al., 2020). Plant species have been employed not only because they are easily accessible and low-cost but also because people believe that natural products are less harmful to health than synthetic medication (Oliveira et al., 2020).

One of the medicinal plants native to Brazil is the species *Psidium myrtoides* (Myrtaceae), also known as *araçá* and *araçá-roxo* in Brazilian Portuguese. It is mostly found in the *Cerrado*, but also in three phytogeographic domains in Brazil: *Caatinga*, *Cerrado* and *Mata Atlântica* (Dias et al., 2022). This plant should be investigated because the species exhibits several biological activities (Dias et al., 2022).

Species that belong to the genus *Psidium* are known because they are rich in characteristics of nutritional interest, such as vitamins and antioxidant compounds. Another relevant issue is the promising antibacterial activity of extracts from plants that belong to this genus (Cordeiro et al., 2020).

Tooth decay has been defined as a disease which results from ecological or metabolic changes in the environment of dental biofilm caused by frequent exposure to fermentable carbohydrates. Thus, there are changes in bacteria that cause tooth decay, i. e., the ones that were balanced and low-cariogenic become unbalanced and high-cariogenic (Batista et al., 2020). The following bacteria were investigated by this study: *Streptococcus mutans, S. sobrinus, S. mitis, S. salivarius, S. sanguinis, Enterococcus faecalis* and *Lactobacillus casei* (Leites et al., 2006). In the search for natural alternatives against cariogenic bacteria, extracts from plants that grow in the Brazilian *Cerrado* are promising anticariogenic agents (Volpato et al., 2022).

Another species under investigation is *Xanthomonas citri*, a phytopathogenic bacterium which causes the disease called citrus canker (Ference et al., 2018). Advanced-stage lesions on leaves, fruit and twigs usually lead to tissue rupture, which enables microorganisms to enter and trigger the rot process (Ference et al., 2018). Chemistry of Natural Products has investigated the potential of essential oils and plant extracts to fight *Xanthomonas* sp., since they may be safe to health and the environment (Braga et al., 2022).

The third and last class of bacteria investigated by this study belongs to the genus *Mycobacterium*. *M. avium*, *M. kansasii* and *M. tuberculosis* are species that cause respiratory infections, but *M. tuberculosis* is the only one which is considered a deadly infectious-contagious bacterium. Besides, *M. avium* and *M. kansasii* are classified into Nontuberculous Mycobacteria (NTB) since their characteristics are different from the ones exhibited by species that belong to the *M. tuberculosis* complex (Carneiro et al., 2018). NTB tend to cause lung disorders when they associate with other factors, such as chronic obstructive pulmonary disease, previous tuberculosis, cystic fibrosis, bronchiectasis, HIV and organ transplants. It has been known that abnormalities in the lung structure and immunosuppression conditions favor the development of lung disease by NTB (Carneiro et al., 2018). The most common symptoms caused by the three species under evaluation are chronic cough, weight loss and fever.

In this scenario, plants have been an important source of natural compounds that exhibit antimycobacterial activity, a relevant fact since the discovery of new drugs to treat tuberculosis has been a great challenge (Arruda et al., 2012). Mycobacteria are slow-growing pathogenic organisms whose lipid-rich cell walls represent actual protection against aggressive agents; thus, they are considered very resistant strains (Arruda et al., 2012).

Therefore, this study aimed at evaluating, for the first time, *in vitro* anticariogenic, anti-*Xanthomonas citri* and antimycobacterial of hexane extracts from *P. myrtoides* O. Berg dry leaves – collected in both dry and rainy seasons – and dry pericarp of unripe fruit (Figure 1).

Figure 1– *P. myrtoides* O. Berg twigs, leaves and unripe fruit

Source: Authors (2024)

2 MATERIAL AND METHODS

2.1 Plant material

Psidium myrtoides O. Berg (Myrtaceae) leaves and unripe fruit were collected at the Instituto Federal Goiano - Campus Rio Verde, in Rio Verde (16°06'20"S and 51°17'11"W), Goiás (GO), Brazil. Leaves were collected in two different seasons, i. e., the dry one (average temperature and precipitation were 30.6 °C and 1.5 mm, respectively, from June to August 2017) and the rainy one (average temperature and precipitation were 29.7 °C and 178 mm, respectively, from January to March 2018). Plant material was collected in tropical climate in a region of the Brazilian *Cerrado* whose soil is dark red latosol with clay and sandy-clay textures. The plant was identified by the botanist Luzia Francisca de Souza and a voucher specimen of *P. myrtoides* (HJ998) was deposited at the Herbarium Jataiense Professor Germano Guarim Neto.

2.2 Preparation of hexane extracts

Leaves (457 g – rainy season and 400 g – dry season) and pericarp of unripe fruit (377 g) were dried in an oven at 40 °C and ground in a Wiley mill. Subsequently, they were exhaustively cold-extracted with hexane for five days. Extracts were then filtered and concentrated under reduced pressure, resulting in 15-g dry leaf (dry season), 12-g dry leaf (rainy season) and 8-g dry pericarp hexane extracts.

2.3 Anticariogenic activity

In vitro antibacterial activity of hexane extracts was determined by Minimum Inhibitory Concentration (MIC) assays which were based on the broth microdilution method (Lemes et al., 2018). *Streptococcus salivarius* (ATCC 25975), *Streptococcus sobrinus* (ATCC 33478), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguinis* (ATCC 10556), *Lactobacillus casei* (ATCC 11578) and *Enterococcus faecalis* (ATCC 4032) were the standard strains in the assays. Firstly, bacteria were transferred to blood agar (Difco Labs, Detroit, MI, USA) and individual 24-h colonies were suspended in 10.0 mL tryptic soy broth (Difco). A spectrophotometer (Femto, São Paulo, SP, Brazil) at wavelength (λ) of 625 nm was used for standardizing suspensions of every microorganism so as to match transmittance of 81, equivalent to 0.5 in the McFarland scale (1.5 x 10^8 CFU/mL). Dilution of the standardized suspension generated final concentration of 5 x 10^5 CFU/mL. Hexane extracts were dissolved in DMSO (Merck, Darmstadt, Germany) at 16.0 mg/mL. Concentrations ranging from 400 to 0.115 μg/mL were achieved after dilution of extracts in tryptic soy broth (Difco). After dilution, DMSO concentrations ranged between 4 % and 0.0039 % (v/v). Negative controls, three wells inoculated with DMSO at concentrations ranging from 4 % to 1 % and one non-inoculated well, with no antimicrobial agent, were included. An inoculated well helped to evaluate whether the broth was adequate for microorganisms to grow. The positive control was chlorhexidine (CH) at concentrations ranging from 5.9 to 0.115 μg/mL, diluted in tryptic soy broth (Difco). Ninety-six-well microplates were sealed with parafilm and incubated at 37 °C for 24 h. After that, 30 mL of an aqueous solution with 0.02 % resazurin (Sigma-Aldrich, St. Louis, MO, USA) was added to every well to show microorganism viability (Lemes et al., 2018; Melo et al., 2017). The lowest concentration of the sample that inhibited microorganism growth (MIC value) was determined as the lowest concentration of hexane extracts that was able to prevent the resazurin solution from changing its color (Lemes et al., 2018; Melo et al., 2017). All assays were conducted in triplicate.

2.4 Anti-*Xanthomonas citri* activity

The bacterium *X. citri* subsp. *citri* (*X. citri* isolated 12, sensitive to copper; *X. citri* isolated 1733, tolerant to copper; and *X. citri* isolated 1647, resistant to copper) was grown in Nutrient Agar (NA) or Nutrient Broth (NB) and incubated at 28°C for 72 hours. All strains were supplied by researcher Dr. Franklin Behlau, Ph. D., at the Fund for Citrus Protection (FUNDECITRUS) located in Araraquara, SP, Brazil.

MIC is the lowest concentration of hexane extracts that was able to inhibit bacterial growth. It was determined by using the broth microdilution method on 96-well culture plates. The methodology recommended by Iantas et al*.,* (2021) was followed with some modifications. Firstly, samples were dissolved in 5% DMSO and then diluted in NB to reach concentrations ranging from 0.98 to 2.000 µg/mL. Inoculums were adjusted to produce final cell concentration of 5×10^5 CFU/mL. Streptomycin was used as positive control at concentrations ranging from 0.0115 to 5.9 µg/mL. Plates were incubated in Biochemical Oxygen Demand (BOD) at 28ºC for 72 h. After incubation, 30 µL resazurin aqueous solution at 0.02% used as bacterial revelator was added to every well. Plates were incubated again at 28◦C for 12 h. Wells that became pink were those where bacterial growth took place while those that remained blue showed that there was inhibition. Three independent experiments were performed in triplicate.

2.5 Antimycobacterial activity

To evaluate the antimycobacterial activity the following American Type Culture Collection (ATCC) and clinical isolates were used: *M. tuberculosis* H37Rv (ATCC 27294), *M. kansasii* (ATCC 12478) and *M. avium* (ATCC 25291) and maintained at -80 °C.

To evaluate the antimycobacterial activity of the samples, the MIC was determined by microdilution in a microplate. Resazurin was used to reveal bacterial growth by the Resazurin Microtiter Assay (REMA) method procedure adapted from Palomino et al. (2002). The experiment was conducted in triplicate. The hexane extracts were dissolved in DMSO and serially diluted in Middlebrook 7H9 broth (Difco, Sparks, MD, USA) before inoculation. The concentrations of the tested extracts ranged from 3.90 to 500 µg/mL, while the final DMSO (Sigma-Aldrich, St. Louis, MO, USA) content in the assay was less than 0.3%. Isoniazid (Sigma-Aldrich) was used as the reference antibiotic drug, was used at concentrations ranging from 0.015 to 1.0 µg/mL. The inoculum was prepared by introducing a range of colonies grown in Ogawa–Kudoh (LaborClin, Pinhais, PR, Brazil) in a tube containing glass beads with 500 µL of sterile water. A 200-µl aliquot was transferred to a tube containing 2 ml of 7H9 broth (Difco), incubated at 37◦C for 7 days and compared with McFarland scale 1. After the inoculum was standardized, it was diluted with 7H9 broth (Difco) at a 1:25 ratio. Growth controls containing no antibiotic and sterility controls without inoculation were also included. The plates were incubated at 37◦C for 7 days. Subsequently, 30 μL of 0.02% resazurin (Sigma-Aldrich) aqueous solution was added to each well. After standing for 18 h, the visual MIC was defined as the lowest samples concentration that was able to inhibit growth of the *Mycobacterium* strains.

3 RESULTS AND DISCUSSION

In vitro assays showed that three hexane extracts exhibited strong antibacterial activity. The one from *P. myrtoides* leaves collected in the dry season (HE-DS) was very active against the following cariogenic bacteria: *Streptococcus mitis* (MIC = 50 μg/mL), *S. sanguinis* (MIC = 50 μg/mL), *S. salivarius* (MIC = 50 μg/mL) and *S. mutans* (MIC = 50 μg/mL). Besides, it was considered active against *S. sobrinus* (MIC = 125 μg/mL), *Lactobacillus casei* (MIC = 400 μg/mL) and *Enterococcus faecalis* (MIC = 400 μg/mL) (Table 1).

Anticariogenic activity exhibited by hexane extract from *P. myrtoides* leaves collected in the rainy season (HE-RS) was similar to the one of HE-DS, except MIC values of *S. salivarius* (MIC = 100 μg/mL) and *S. mutans* (MIC = 100 μg/mL) (Table 1). Hexane extract from pericarp of unripe fruit (HE-PP) exhibited the highest MIC values: *S. mitis* (MIC = 400 μg/mL), *S. sanguinis* (MIC = 200 μg/mL), *L. casei* (MIC = 200 μg/mL), *E. faecalis* (MIC = 400 μg/mL), *S. salivarius* (MIC = 250 μg/mL), *S. sobrinus* (MIC = 250 μg/mL) and *S. mutans* (MIC = 400 μg/mL) (Table 1).

MIC values of hexane extracts from *P. myrtoides* are worth mentioning since the literature has reported the following concentration ranges and their activities: samples whose MIC values are below 100 μg/mL, between 101 and 500 μg/mL, between 501 and 1500 μg/mL and between 1500 and 2000 μg/mL are considered very active, active, moderately active and weakly active, respectively. MIC values above 2000 µg/mL denote inactive samples (Carvalho et al., 2022).

Table 1 – Anticariogenic activity of hexane extracts from *P. myrtoides* (MIC = µg/mL)

Bacteria	HE-DS	HE-RS	HE-PP	CH
Streptococcus mitis (ATCC 49456)	50	50	400	1.47
S. sanguinis (ATCC 10556)	50	50	200	0.74
S. salivarius (ATCC 25975)	50	100	200	0.74
S. sobrinus (ATCC 33478)	125	125	400	0.18
S. mutans (ATCC 25175)	50	100	250	0.09
Enterococcus faecalis (ATCC 4032)	400	400	250	2.95
Lactobacillus casei (ATCC 11578)	400	400	400	0.37

HE-DS: hexane extract from leaves (dry season); HE-RS: hexane extract from leaves (rainy season); HE-PP: hexane extract from pericarp of unripe fruit; CH: chlorhexidine (positive control)

HP-DS and HE-RS were highly active against important bacteria that cause tooth decay, i. e., *Streptococcus mutans*, *S. salivarius*, *S. sanguinis* and *S. mitis*. *S. mutans* is a major dental pathogen and the main etiological agent of tooth decay. It is capable of fermenting the largest number of carbohydrates, by comparison with the other Gram-positive bacteria known so far (Bittencourt et al., 2022). *S. mitis*, *S. salivarius* and *S. mutans* are considered species that are closely connected to a person's decay indices since there is positive association between their levels and high prevalence of tooth decay (Galrão et al., 2012). *S. sanguinis* also causes tooth decay and periodontitis, diseases that result from dysbiosis of oral microbiomes (Zhu et al., 2018). HE-PP, whose MIC values ranged from 200 to 400 µg/mL, was active against all cariogenic bacteria under investigation.

HE-DS, HE-RS and HE-PP were assayed against *X. citri* in three situations: against *X. citri* (resistant to copper), *X. citri* (tolerant to copper) and *X. citri* (sensitive to copper).

Both hexane extracts exhibited promising activities against bacteria. All MIC values ranged from 31.25 to 100 µg/mL (Table 2).

Table 2 – Anti-*Xanthomonas citri* activity of hexane extracts from *P. myrtoides*

Bacteria	HE-DS	HE-RS	HE-PP	Streptomycin
X. citri 1647 (resistant to copper)	62.5	62.5	100	0.0461
X. citri 1733 (tolerant to copper)	31.25	31.25	100	0.3688
X. citri 12 (sensitive to copper)	100	100	100	0.0922

HE-DS: hexane extract from leaves (dry season); HE-RS: hexane extract from leaves (rainy season); HE-PP: hexane extracts from pericarp of unripe fruit; Streptomycin: positive contro

According to Ríos & Recio (2005) and Gibbons (2008), crude extracts whose MIC values were below or equal to 100 µg/mL are considered promising by studies of new antimicrobial agents. As a result, these parameters were used by this study to determine whether hexane extracts from *P. myrtoides* were promising. Therefore, HE-DS, HE-RS and HE-PP may be considered good sources of active metabolites against *X. citri*.

X. citri causes the phytosanitary problem called citrus canker (Bansal *et al*., 2017). The bacterium invades the vascular tissue of plants, where it multiplies and propagates. Afterwards, throughout leaf or vascular infection, plant cells adjacent to bacterium colonies start to degrade. Plant organelles degenerate, cell walls fragmentate and, finally, bacteria invade and multiply withing plant cells. A typical symptom of contamination is premature fruit and leaf drop (Bansal et al., 2017).

Other bacteria assayed by this study belong to the class of mycobacteria, i.e., *Mycobacterium tuberculosis*, *M. kansasii* and *M. avium*. HE-DS, HE-RS and HE-PP exhibited good antimycobacterial potential since MIC values ranged from 125 to 500 µg/mL (Table 3).

To interpret MIC values, the theoretical basis of this study was proposed by Melo et al. (2017) and Silva et al. (2020) who state that MIC values of 500 µg/mL and 250 µg/mL are considered moderately active and active, respectively, against mycobacteria under evaluation. On the other hand, low MIC values of 125 μg/mL should be highlighted since the literature reports that MIC ≤ 200 μg/mL is remarkable (Cabral et al., 2020). These findings show the high antimycobacterial potential of hexane extracts from *P. myrtoides*.

Table 3 – Antimycobacterial activity of hexane extracts from *P. myrtoides*

Bacteria	HE-DS	HE-RS	HE-PP	Isoniazid
M. tuberculosis (ATCC 27294)	500	250	500	0.06
M. kansasii (ATCC 12478)	500	250	125	0.25
M. avium (ATCC 25291)	125	500	125	>1.0

HE-DP: hexane extracts from leaves (dry season); HE-RP: hexane extracts from leaves (rainy season); HE-PP: hexane extracts from unripe fruit pericarp; Isoniazid: positive control

Chemical and biological studies of plant extracts have been efficient alternatives in the discovery of new bioactive compounds. The main factor that triggers the need to search for new bacterial agents is constant development of bacterial resistance against conventional antibiotics (Vaou et al., 2021). Therefore, new drugs have been found as the result of advances in identification of new natural antibiotic sources and knowledge about chemical diversity of plants in terms of their secondary metabolites (Vaou et al., 2021). In addition, the following mechanisms of action of secondary metabolites against different bacteria have been known: inhibition of bacterial protein biosynthesis, inhibition of DNA synthesis and function and disruption of cell and structure (Vaou et al., 2021).

In sum, this study contributes to the literature by adding new information on the chemical and biological potential of hexane extracts from *P. myrtoides*. So far, *P. myrtoides* has been known as the species that produces active essential oils against different species of fungi, parasites and bacteria, such as *Colletotrichum gloeosporioides,* *Candida glabrata, Candida albicans, Streptococcus sanguinis, Staphylococcus aureus, Leishmania amazonensis and Trypanossoma cruzi* (Freitas et al., 2021; Dias et al., 2022; Dias et al., 2022a).

4 CONCLUSIONS

The antibacterial screening carried out by this study showed the promising potential of hexane extracts from *P. myrtoides* leaves – collected in both dry and rainy seasons – and pericarp of unripe fruit. All three hexane extracts under investigation proved to be active against several bacterial strains. It should be highlighted that the main advantage to use plant extracts is that they are sources of new compounds which pathogens cannot inactivate. Besides, they are less toxic, easily degraded by the environment, derived from renewable resources and have a broad mechanism of action. However, further studies are needed to isolate active secondary metabolites found in the extracts under evaluation and to assay them *in vitro* and *in vivo*.

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