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Original Paper

Green propolis as an adjuvant against nontuberculous mycobacteria

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Abstract

Natural products have been touted as important tools because of their vast potential for the development of compounds with antimicrobial activity and the possible inhibitory activity and/or adjuvant resistance mechanisms. Propolis has been empirically used for many years for the treatment of diseases, mainly due to its antioxidant, anti inflammatory and antimicrobial activities. This study aimed to evaluate the *in vitro* antimycobacterial activity of the ethanol extract of propolis alone and in combination with rifampicin (RIF), amikacin (AMI) and ciprofloxacin (CIP). The ethanol extract of propolis showed antibacterial activity against *Mycobacterium chelonae* and *M. kansasii* and was capable of increasing AMI, RIF and CIP activity in combination. On the other hand, compared to *M. absecessus*, *M. fortuitum* and *M. avium*, the extract was not active at 200 µg/mL and did not show pronounced adjuvant capacity when evaluated in association with the drugs. Based on these results, it can be concluded that the ethanol extract of propolis could be an alternative in the development of new drugs and can be used complementary with the current mycobacteriosis treatment.
Key words: additivity, antimicrobial, *Mycobacterium* sp., propolis.

Resumo

Os produtos naturais têm sido apontados como ferramentas importantes devido ao seu vasto potencial para o desenvolvimento de compostos com atividade antimicrobiana e a possível atividade inibitória e/ou adjuvante de mecanismos de resistência. O própolis é utilizado empiricamente há muitos anos no tratamento de doenças, principalmente devido às suas atividades antioxidantes, anti-inflamatórias e antimicrobianas. Este estudo teve como objetivo avaliar a atividade antimicobacteriana *in vitro* do extrato etanólico de própolis isoladamente e em associação com rifampicina (RIF), amicacina (AMI) e ciprofloxacina (CIP). O extrato etanólico de própolis mostrou atividade antibacteriana frente *Mycobacterium chelonae* e *M. kansasii* e foi capaz de aumentar a atividade de AMI, RIF e CIP em associação. Por outro lado, frente a *M. absecessus*, *M. fortuitum* e *M. avium*, o extrato não foi ativo a 200 µg/mL e não apresentou capacidade adjuvante pronunciada quando avaliado em associação com os fármacos. Com base nesses resultados, pode-se concluir que o extrato etanólico de própolis pode ser uma alternativa no desenvolvimento de novos fármacos e pode ser utilizado complementarmente com o atual tratamento das micobacterioses.
Palavras-chave: aditividade, antimicrobiano, *Mycobacterium* sp., própolis.

Introduction

The genus *Mycobacterium* consists of a wide variety of organisms, including obligate, opportunistic pathogens and saprophytic species (Falkinham 2016). Nontuberculous mycobacteria (NTM) are opportunistic microorganisms and may occasionally cause serious diseases in humans, being the most frequent and commonly related to patients with preexisting pulmonary infections or compromised immune systems and attributed

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mainly to members of the *Mycobacterium avium* complex and *M. abscessus* complex (Wu *et al*. 2018).

Treatment of NTM usually occurs with the same antimicrobials used to treat tuberculosis since the diagnosis of NTM is often difficult and is supposedly treated as TB, although there are differences in the symptoms (Egelund *et al*. 2015). However, NTM is generally resistant to conventional tuberculostatic drugs, which may compromise the therapeutic response since the mechanisms of drug susceptibility in NTM are distinct from *M. tuberculosis*, and variations in the susceptibility of some antimycobacterial agents may occur with the species (Wu *et al*. 2018).

In addition, Ali *et al*. (2018) demonstrated that extracts of propolis have inhibitory activity against *Mycobacterium tuberculosis*, potentializing the effect of the main drugs used for the treatment of TB. Historically, the discovery of antimycobacterial drugs and preclinical testing efforts have been almost uniquely centered on *M. tuberculosis*, with virtually no concentrated effort toward extended-spectrum agents that cover NTM, yet represents a therapeutic challenge, as current and innovative treatment options for NTM are limited or unavailable (Kasperbauer & De Groote 2015; Wu *et al*. 2018).

This study aimed to evaluate the *in vitro* antimicrobial activity of the ethanolic extract of green propolis and its combined effect with rifampicin, amikacin, and ciprofloxacin against five mycobacterial species that cause pulmonary infection.

Material and Methods

Propolis sample and Propolis Extract Preparation

The green propolis sample was obtained by Nectar Farmacêutica Ltda. in Minas Gerais (Brazil) and conditioned at -20 ºC. The extract was prepared previously as described by Paulino *et al.* (2002). The sample was frozen and macerated with extraction solution containing absolute ethanol and stirred at 37 °C for seven days. After the solvent was evaporated, the dry matter was dissolved in PBS (pH 6.2) at a final concentration of 40 mg/mL.

Isolation and preparation of the inoculum

The experiments were conducted at the Medical Microbiology Research Center at the Federal University of Rio Grande (FURG), Rio Grande/RS, Brazil. The strains of *Mycobacterium chelonae* (ATCC 946), *M. abscessus* (ATCC 19977), *M. fortuitum* (ATCC 35931), *M. avium* (ATCC 03057 HC) and *M. kansasii* (ATCC 12478) were kept in Ogawa-Kudoh medium for approximately 14 days. Bacterial suspensions were prepared in sterile water tubes containing glass beads. The suspension was homogenized by vortexing, and the turbidity was adjusted according to scale 1 McFarland (3.2×10^6 CFU/mL). The inoculum was prepared with a 1:20 bacterial suspension in 7H9 medium (Middlebrook) (Palomino *et al*. 2002).

Evaluation of minimum inhibitory concentration (MIC) of extract and antibiotics

The method used to determine the antimycobacterial activity was the Resazurin microtiter assay (REMA), using 96-well microplates (Palomino *et al*. 2002). At the periphery, 200 µL of sterile distilled water was added to avoid evaporation during the incubation period (7–9 days). Then, 100 µL of 7H9 medium enriched with 10% OADC (oleic acid, albumin, dextrose and catalase) was added to the each well, and 100 µL of the propolis ethanolic extract at the initial concentration of 200 µg/mL or antibiotics amikacin, rifampicin and ciprofloxacin at the initial concentration of 10 µg/mL were added. A 1:2 microdilution was performed, where the concentrations ranged from 200 µg/mL to 6.25 µg/mL for propolis and 10 µg/mL to 0.03 µg/mL for antibiotics. After microdilution, 100 µL of the bacterial inoculum was added as described above. The microplate was incubated according to the growth time of each strain: for the slow-growing mycobacteria (*M. kansasii* and *M. avium*), seven days, and for the fast growing bacteria (*M. abscessus*, *M. chelonae* and *M. fortuitum*), five days. After the incubation period, 30 µL of 0.02% resazurin, which acts as an indicator of cell viability, was added, and incubated again for 48 h. MIC was defined as the minimum concentration capable of inhibiting bacterial growth.

Determination of the interaction between the ethanolic extract of green propolis with antibiotics by the checkerboard method

After MIC determination, the effect of the combination of ethanolic propolis extract with

rifampicin, amikacin and ciprofloxacin was determined based on the checkerboard method described by Caleffi-Ferracioli *et al*. (2013). For each bacterial isolate (*M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. avium* and *M. kansasii*), a microplate was used with the association of the propolis extract and an antimicrobial: rifampicin, amikacin or ciprofloxacin. Fifty microliters of 10% OADC-enriched 7H9 medium was added to all test wells; 50 µL of the antimicrobial was added, at the initial concentration according to the MIC of each one, and a serial dilution 1:2 was performed in the Y axis. Subsequently, 50 µL of the propolis extract previously diluted 1:2, at the initial concentration of 200 µg/mL, was added in each column of X axis. Finally, 100 µL of the bacterial inoculum was added. The plate was incubated in a bacteriological incubator for 5–7 days, and then 30 µL of 0.02% resazurin, which acts as an indicator of cell viability through an oxi-reduction reaction, was added and incubated again for 48 h. The fractional inhibitory concentration index (FICI) was defined as the lowest concentration at which the extract and the antimicrobial in combination were able to inhibit bacterial growth. The interpretation of the results of the checkerboard was performed through the FICI index obtained by the following formula: FICI = (MIC of the combined extract/ MIC of the extract alone) + (MIC of the combined antimicrobial/MIC of the antimicrobial alone).

The FICI results were interpreted as follows: FICI ≤ 0.5 = SYNERGISM; 0.5 < FICI ≤ 1 = ADDITIVITY; 1 < FICI ≤ 2 = INDIFFERENCE and FICI > 2 = ANTAGONISM. In addition, the modulatory factor was calculated as follows: MIC of antibiotic alone/MIC of antibiotic in combination with propolis (Roell *et al*. 2017).

Determination of total phenolic content (TPC)

The quantification of total phenols was carried out according to the methodology described by Pires *et al*. (2017), in microplate, using the Folin-Ciocalteau reagent. In brief, the extract sample was diluted with methanol from 100 to 0.78 µg/mL in microtiter plates. For TPC analysis, a calibration curve was established using gallic acid, and the absorbance was measured at λ = 760 nm. The TPC results was expressed as milligrams of gallic acid equivalent per gram of propolis sample.

Results and Discussion

The antimycobacterial activity of green propolis against NTM (*M. kansasii*, *M. avium*, *M fortuitum*, *M. abscessus*, *M. chelonae*) was different according to the species of mycobacteria evaluated (Tab. 1). When evaluated against *M. avium*, *M. abscessus* and *M. fortuitum*, the extract showed no antimycobacterial activity (MIC > 200 µg/ mL); however, the extract was active against *M. chelonae* and *M. kansasii*, with an MIC of 25 µg/ mL. In addition, at 200 µg/mL, the alcoholic extract of green propolis was not active against strains of *Escherichia coli* and *Klebsiella pneumoniae* and did not present adjuvant activity when evaluated in combination with cephalosporins (data not shown). The antimicrobial activity of different propolis extracts has been widely investigated against several bacteria, especially against strains of *Staphylococcus aureus* (Fernandes *et al*. 2005; Lavinas *et al*. 2019); these studies indicate that the activity of propolis extracts may have a narrow spectrum of related microbial species, mainly gram-positive and mycobacterial microorganisms (Al-Waili *et al*. 2012; Fernandes *et al*. 2005;

Table 1 – Minimum inhibitory concentration (µg/mL) of antibiotics and ethanolic extract of green propolis against five mycobacteria.

	Minimum inhibitory concentration (µg/mL)			
	Amikacin	**Rifampicin**	**Ciprofloxacin**	**Propolis extract**
Mycobacterium chelonae	≤ 0.5	1	1	25
Mycobacterium abscessus	16	> 128	32	> 200
Mycobacterium fortuitum	0.5	2	0.03	> 200
Mycobacterium kansasii	0.06	0.015	0.5	25
Mycobacterium avium	2	≤ 0.25	2	> 200

Stepanović *et al*. 2003; Wojtyczka *et al*. 2013), similar to that reported in our study.

The ethanolic extract of green propolis showed antimicrobial activity, with MIC = 25 µg/mL, against *M. chelonae* and *M. kansasii*, when tested alone, and a modulatory effect capable of decreasing the MIC values of amikacin and ciprofloxacin eight and four times, respectively. However, when combined with the antimicrobials evaluated, propolis had an additive effect against *M. abscessus* (amikacin/propolis), *M. chelonae* and *M. avium* (rifampicin/propolis). These results indicate that despite not having synergistic effects, the ethanolic extract of green propolis presented modulating activity, potentiating the antimicrobial activity of these drugs.

Together, these data suggest that propolis may potentiate the effect of some antibiotics, especially those whose mechanism of action is associated with genetic processes that interfere with the maintenance of microbial species. In addition, amikacin has been recommended in adjuvant therapy for NTM infections with significant bactericidal activity (Davis *et al*. 2007), and in this work, amikacin and the extract displayed only positive associations (indifferent or additive) independent of the evaluated mycobacterial species; therefore, green propolis extract is a good model for the development of new therapeutic alternatives to be used with the current NTM treatment to reduce doses and toxicity and to enable the introduction of a new chemical class in the available pharmacological arsenal. In addition, studies of the chemical composition, activity studies and *in vivo* toxicity of these substances would be of great interest as a continuation of this work.

The antimicrobial activity found could be related to phenolic compounds that have been identified as one of the main constituents of Brazilian propolis and responsible for the bioactive activity of this natural product against a series of pathogenic microorganisms (Zabaiou *et al*. 2017). The ethanolic extract of green propolis had a total phenolic content (measured as gallic acid equivalents) of 448.80 mg/g (Fig. 1). The findings of this study coincide with that described by the literature for propolis, since phenolic acid has been identified as the main classes of secondary metabolites extracted in propolis ethanolic and the antimicrobial activity may be due to the action of the phenolic compounds detected (Lavinas *et al*. 2019; Machado *et al*. 2016), being appointed as responsible for inhibit NTM growth in previous studies by Mickymaray *et al*. (2020) and Przybyłek & Karpiński (2019).

The chemical constituents of propolis, as well as its varied activity, especially green propolis, have been described in the literature; however, the antimycobacterial activity against NMT has not yet been investigated (Al-Waili *et al*. 2012; Fernandes *et al*. 2005; Franchin *et al*. 2018; Pasupuleti *et al*. 2017; Scheller *et al*. 1998, 1999, Stepanović *et al*. 2003; Wojtyczka *et al*. 2013; Yildirim *et al*. 2004). Some phenolic compounds, such as isoflavanoids and phenolic acids, have demonstrated the potential to inhibit the mycobacterial efflux system in NTMs (Gröblacher *et al.* 2012; Lechner *et al.* 2008).

In addition, it was previously identified that the antibacterial activity of propolis could be influenced by the variation in chemical composition, which is closely related to the geographic, climatic aspects and the associated plant species (Al-Waili *et al*. 2012; Zabaiou *et al*. 2017). This fact could be corroborated by the findings of Monzote *et al*. (2012), which identified the antimicrobial activity of three Cuban propolis, known as brown, red and yellow, against different microorganisms, among them *S. aureus* and *E. coli*. This fact was in contrast to the results found in our study, where the object of study was green propolis, which presented activity strictly related to nontuberculous mycobacteria.

Antimicrobial activity of aqueous extracts of Turkish propolis has been identified by *in vivo* assays using guinea pigs infected with *M. tuberculosis* (Yildirim *et al*. 2004), whereas Scheller *et al*. (1998) identified similar anti-TB

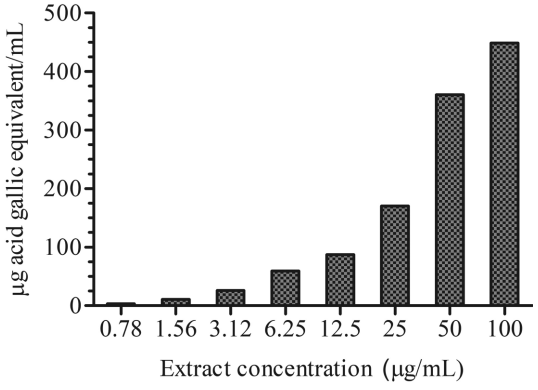

Figure 1 – Values of ethanolic extract concentration (µg/mL) and total phenols (µg of gallic acid/mL of the extract) contents of Brazilian green propolis.

activity in ethanolic extracts, corroborating our findings regarding the genus *Mycobacterium*.

Mycobacterium kansasii continues to be the most easily treatable NTM lung disease pathogen, and unlike most NTM, there is a good correlation between *in vitro* and *in vivo* susceptibility in response to a variety of antimicrobial agents, including rifampicin, macrolides and fluoroquinolones Griffith *et al*. (2007), which was also evidenced in our study, where *M. kansasii* presented the lowest MIC values for amikacin (0.06 µg/mL) and rifampicin (0.015 µg/mL) between the NTM species evaluated.

Different species within the same genus of microorganisms differ in their susceptibility to antimicrobials. This susceptibility is a reflection of the genetics of each species and environmental pressures (Fogelson *et al.* 2019). As can be seen in Table 2, rifampicin increases its inhibitory effect in *M. chelonae* and *M. avium* when combined with the propolis ethanolic extract. In contrast, the opposite occurs with *M. fortuitum*, where we observed an antagonistic effect in this same combination. The fact is that the first two species mentioned are phylogenetically distant from *M. fortuitum* (Tortoli *et al.* 2017), which may be linked to the differences observed in the susceptibility pattern. Still in Table 2, we can see that the reverse occurs for the combination ciprofloxacin + propolis, where it is antagonistic against the growth of *M. chelonae* and *M. avium*, but demonstrates an additivity profile when tested against *M. fortuitum*.

In relation to the antimicrobial FICI and the ethanolic extract of green propolis (Table 2), amikacin had an additive effect only when evaluated in combination with *M. abscessus*. However, analysis of modulatory factors (MFs) indicated that the MIC value of this antibiotic against *M. chelonae* was reduced 8.3 times, unlike *M. abscessus* and *M. kansasii*, which had a reduction of only half the value.

Compounds that, through rational synergism, act to facilitate the action of antimycobacterial drugs with actions at the intracellular level, interfering in processes critical for maintenance and viability, such that, for example, they aid in the permeability of these drugs, are needed (Falkinham 2018). A similar effect of synergism between propolis and antimicrobial drugs that act on the bacterial ribosome (such as amikacin) has been demonstrated in a previous study (Maurer *et al.* 2014; Orsi *et al.* 2012), in addition to the capacity of ethanolic extract of Brazilian green propolis to increase the immunological response acting as adjuvant in the fight against inflammatory processes (Franchin *et al.* 2018).

Rifampicins have broad antimicrobial coverage and are frequently used in the treatment of NTM infections (Ramis *et al.* 2018), presenting an additive interaction with the ethanolic extract of green propolis for *M. chelonae* and *M. avium*, similar to the findings reported by (Scheller *et al*. 1999), which showed a positive interaction between the association of this antimicrobial with the ethanol extract of propolis against other mycobacteria.

Ciprofloxacin belongs to the group of fluoroquinolones, which have been used in the treatment of NTM infections (Egelund *et al*. 2015). Based on results of the present study, the combination of ciprofloxacin and the ethanolic extract of green propolis, even with the indifference

Table 2 – Combined minimum inhibitory concentration (MIC), fractional inhibitory concentration index (FICI) and modulatory factors (MFs) of antibiotics and ethanolic green propolis extract against fast-growing and slow-growing mycobacteria (µg/mL).

Strains	MIC (µg/mL) Amikacin/ Propolis	FICI	MF	MIC (µg/mL) Rifampicin/ Propolis	FICI	MF	MIC (µg/mL) Ciprofloxacin/ Propolis	FICI	MF
Mycobacterium chelonae	0.06 / 25	1.12	8.33	0.5 / 25	1	2	0.5 / 50	3	1
Mycobacterium abscessus	8 / 100	1	2	> 128 / > 200	2	1	> 128 / > 200	5	0.25
Mycobacterium fortuitum	0.5 / 25	1.1	1	> 128 / > 200	5	0.25	0.03 / 6.25	1.0	1
Mycobacterium kansasii	0.03 / 12.5	1.5	2	0.015 / 6.25	1.2	1	0.12 / 25	1.2	4.16
Mycobacterium avium	2 / 25	1.1	1	≤ 0.25 / 6.25	1.0	1	4 / 50	2.3	0.5

MIC = minimum inhibitory concentration; FICI = fractional inhibitory concentration index; MF = modulatory factor; FICI ≤ 0.5 = synergism; 0.5 < FICI ≤ 1 = additivity; 1 < FICI ≤ 2 = indifference; FICI > 2 = antagonism.

identified by FICI for *M. kansasii*, there seems to be a positive association since the modulatory factor was 4.16.

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