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Chemical characterization and *in vitro* antimicrobial activity of honeybee propolis and *Scaptotrigona jujuyensis* geopropolis against tomato pathogenic bacteria

Caracterização química e atividade antimicrobiana *in vitro* de própolis de abelhas e de geoprópolis de *Scaptotrigona jujuyensis* contra bactérias patogênicas do tomate

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Highlights:
Propolis and geopropolis significantly differed in their chemical characteristics.
HPLC-DAD revealed distinctive profiles between the hydroalcoholic extracts.
Propolis exhibited higher antibacterial activity than geopropolis.
Antibacterial activity of hydroalcoholic extracts was dose-dependent.
The differences in chemical composition, mainly in total polyphenol content and dry extract, were related to the antibacterial effects.

Abstract

The antimicrobial activity of four concentrations of hydroalcoholic extracts from honeybee propolis and *Scaptotrigona jujuyensis* geopropolis was screened *in vitro* against five tomato pathogenic bacteria. The agar-well diffusion method was used and the tested bacteria were *Clavibacter michiganensis* subsp. *michiganensis*, *Xanthomonas gardneri*, *Xanthomonas vesicatoria*, *Pseudomonas corrugata*, and *Pseudomonas mediterranea*. The main chemical characteristics of propolis and geopropolis, including the polyphenol profile through HPLC-DAD, were also determined. Geopropolis raw sample presented higher values of moisture (7.78%), waxes (50.79%) and ashes (3.69%) than propolis (4.59%, 31.16% and 2.42% respectively). The total polyphenol content and the dry extract were higher in propolis hydroalcoholic extract (3.83 mg eq galic acid mL^{-1} and 7.87%, respectively) than in the extract of geopropolis (0.16 mg eq galic acid mL^{-1} and 0.15%, respectively). Chromatographic analysis confirmed

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the presence of caffeic acid, quercetin, 1,5,7-trihydroxy-flavanone, apigenin, pinobanksin, chrysin, pinocembrin, and galangin in both extracts. The antimicrobial assay showed significant differences between the hydroalcoholic extract activities, as well as between the sensitivity of the tested bacteria. Propolis hydroalcoholic extract dilutions had an inhibitory effect over four of the five tested bacteria, while geopropolis hydroalcoholic extract dilutions were only effective against *C. michiganensis* subsp. *michiganensis*, and to a lesser extent. The sequence of bacteria sensitivity to propolis treatments was: *C. michiganensis* subsp. *michiganensis* > *X. gardneri* > *X. vesicatoria* > *P. corrugata*. *Pseudomonas mediterranea* was not sensitive to any of the hydroalcoholic extracts. The antimicrobial activity of both extracts was dose-dependent where the most concentrated treatments were the most effective (15.0 mg mL^{-1} of geopropolis and 78.7 mg mL^{-1} of propolis dry extract, respectively). The polyphenol content and the HPLC-DAD profile of the hydroalcoholic extracts disclosed differences in chemical composition that helped to explain the outcomes of the *in vitro* assay. These results are a contribution to the characterization of bee bioactive products, specifically to propolis and geopropolis. This study indicates the likelihood of using propolis as a non-conventional strategy to control tomato bacterial diseases.

Key words: *Apis mellifera*. HPLC-DAD. *Scaptotrigona jujuyensis*. Tomato phytopathogens. Total polyphenol content.

Resumo

A atividade antimicrobiana de quatro concentrações de extratos hidroalcoólicos de própolis de abelha e de geoprópolis de *Scaptotrigona jujuyensis* foi avaliada *in vitro* contra cinco bactérias patogênicas do tomate. Foi utilizado o método de difusão em ágar e as bactérias testadas foram *Clavibacter michiganensis* subsp. *michiganensis*, *Xanthomonas gardneri*, *Xanthomonas vesicatoria*, *Pseudomonas corrugata*, e *Pseudomonas mediterranea*. As principais características químicas da própolis e da geoprópolis, incluindo o perfil de polifenóis por meio de HPLC-DAD, foram também determinados. A amostra bruta de geoprópolis apresentou mais altos valores de umidade (7,78%), ceras (50,79%) e cinzas (3,69%) do que a amostra de própolis (4,59%, 31,16% e 2,42%, respectivamente). O conteúdo de fenólicos totais e do extrato seco foi superior no extrato hidroalcoólico de própolis (3,83 mg eq ácido gálico ml^{-1} e 7,87%, respectivamente) do que no extrato hidroalcoólico de geoprópolis (0,16 mg eq ácido gálico ml^{-1} e 0,15%, respectivamente). A análise cromatográfica confirmou a presença de ácido cafeico, quercetina, 1,5,7-tri-hidroxi-flavanona, apigenina, pinobaksina, crisina, pinocembrina, e galangina em ambos os extratos. O ensaio antibacteriano mostrou diferenças significativas entre as atividades dos extratos hidroalcoólicos assim como entre a sensibilidade das bactérias testadas. As diluições do extrato hidroalcoólico de própolis tiveram efeito inibitório sobre quatro das cinco bactérias testadas enquanto as diluições do extrato hidroalcoólico de geoprópolis foram eficientes somente contra *C. michiganensis* subsp. *michiganensis* e em menor extensão. A sequência de sensibilidade das bactérias aos tratamentos com própolis foi: *C. michiganensis* subsp. *michiganensis* > *X. gardneri* > *X. vesicatoria* > *P. corrugata*. *Pseudomonas mediterranea* não foi sensível a nenhum dos extratos hidroalcoólicos. A atividade antibacteriana de ambos os extratos foi dependente da dose, sendo que os tratamentos com maior concentração foram os mais efetivos (15,0 mg mL^{-1} de geoprópolis e 78,7 mg mL^{-1} de própolis, extrato seco, respectivamente). O conteúdo de polifenóis e o perfil HPLC-DAD dos extratos hidroalcoólicos mostraram diferenças na composição química, as quais podem ajudar a explicar os resultados do ensaio *in vitro*. Estes resultados contribuem para a caracterização de produtos bioativos de abelhas, especificamente própolis e geoprópolis. O presente estudo indica a possibilidade de usar própolis como uma estratégia não-convencional para controlar doenças do tomate causadas por bactérias.

Palavras-chave: *Apis mellifera*. Conteúdo de fenólicos totais. Doenças do tomate. HPLC-DAD. *Scaptotrigona jujuyensis*.

Bacterial diseases are a worldwide problem in tomato (*Solanum lycopersicum* L.) production. The use of synthetic pesticides and copper compounds to control the major pathogens affecting this crop has been questioned because of detrimental effects on the environment and humans, plant toxicity, and the necessity of frequent applications. In addition, only a few methods are available in organic agriculture to protect tomato cultivation (Balestra, Heydari, Ceccarelli, Ovidi, & Quattrucci, 2009). Thus, some bioactive natural substances such as propolis have been proposed as alternative treatments to control plant pathogens.

Propolis is an extremely complex resinous material elaborated by honey bees (*Apis mellifera* L.) by mixing their own secretions such as saliva and wax with different plant sources. In general, it is composed of around of 50% resins, 30% waxes, 10% essential oils, 5% pollen, and 5% various organic compounds. The main identified bioactive substances belong to the groups of polyphenols, aromatic acids, and diterpenic acids. This composition is quite variable depending on the origin of the samples which is strongly related to the flora surrounding the hives. Current applications of propolis include numerous biomedical uses, such as wound healing, treatment of burns, acne, and herpes, and dermatitis (Wagh, 2013). However, the use of this natural product to control plant pathogens has been poorly studied.

Although literature has been focused on propolis collected by honey bees, there are numerous species of stingless bees that elaborate a similar material known as geopropolis. This product has been poorly studied and little is known about its chemical composition and its biological activity. Geopropolis is made of plant resin and beeswax, and also presents a high proportion of plant waxes and soil in its constitution. The result of this mixture is a less malleable resinous material when compared to propolis. Despite differences in composition, geopropolis displays similar functions in the hive (Lavinas et al., 2019). In recent years, this product has shown different therapeutic properties such as anti-tumoural, anti-oxidant, anti-inflammatory, gastro-protective, and anti-microbial (Mendes Araújo et al., 2015).

In this context, we proposed to evaluate and compare propolis and geopropolis as new non-conventional alternatives to control tomato bacterial pathogens. Hence, the antimicrobial activity of honeybee propolis and *Scaptotrigona jujuyensis* geopropolis was analyzed *in vitro* by the agar-well diffusion method against five tomato plant pathogenic bacteria: *Clavibacter michiganensis* subsp. *michiganensis, Xanthomonas gardneri*, *Xanthomonas vesicatoria, Pseudomonas corrugata,* and *Pseudomonas mediterranea*. The main chemical characteristics including the polyphenol profile through HPLC-DAD of both types of propolis were also determined.

Propolis (P) from *A. mellifera* was collected from hives located in Río Negro Province, Argentina (36°09′02″S and 70°23′47″W) where the predominant tree species near the apiaries were *Populus* sp., *Salix* sp., *Malus domestica*, *Prunus persica*, and *Pyrus communis*. Geopropolis (GP) from *S. jujuyensis* was obtained from hives located in Formosa Province, Argentina (26°18′32″S and 59°22′20″W). Ecosystems of this region include *Schinopsis balansae*, *Aspidosperma quebracho-blanco*, *Prosopis* sp., *Caesalpinia paraguariensis*, *Salix* sp., and *Schinus molle*. Both samples were stored in sterile bottles, frozen at -15±2 °C and protected from light until use. Hydroalcoholic extracts of each type of propolis (EH-P and EH-GP) were prepared by dissolving 15 g of powdered sample in 150 mL of ethanol:water (70:30 v/v), agitated (300 rpm) for 24 h at room temperature and filtered with Whatman Nº 40 paper (Fernandez et al., 2019).

The chemical characterization of P and GP included moisture, ashes, and waxes as described by Cibanal, Fernández, Krepper, Pellegrini and Gallez (2019) with minor modifications. Briefly, the percent of moisture was calculated after drying

five replicates per sample of 1 g at 105 °C for 48 h. Ash content was determined by calcination at 550 °C for 48 h. Waxes were extracted in a Soxhlet equipment for 24 h at a maximum temperature of 60 °C. The total polyphenol content by the Folin-Ciocalteu method and the dry extract of EH-P and EH-GP were determined (Bedascarrasbure, Maldonado, Fierro Morales, & Alvarez, 2006). Both extracts were analysed by high performance liquid chromatography equipped with a diode array detector (HPLC-DAD) to achieve the chemical polyphenol profile. An HPLC Prominence LC-20A series (Shimadzu Corporation, Japan), equipped with auto-sampler, quaternary pump, and diode array detector (SPD-M20A) was used for this purpose. Separation of each EH sample was carried out on a Phenomenex C-18 column (250 x 4.6 mm i.d., particle size 5 **µm**) using mobile phase water with 0.1% TFA (A) and acetonitrile with 0.1% TFA (B). The injected volume was 10 **µ**l. The following gradients of mobile phase were used at a flow rate of 0.7 mL/min: 35% B, 0 min; 50% B, 10 min; 50% B, 15 min; 80% B, 40 min; 35% B, 50 min; 35% B, 55min. The main components were identified based on a comparison of the retention times and the UV-spectrum with those of the standards.

The bacterial strains used in this study were isolated from diseased tomato plants collected in Uruguay. *Xanthomonas gardneri* (Acc. Nr. MT103547), *X. vesicatoria* (Acc. Nr. MT103548), *Pseudomonas corrugata* (Acc. Nr. MT103549) and *P. mediterranea* (Acc. Nr. MT103550) strains were identified by the sequence of the partial gene *gyr*B using the primers UP-1EF and APrUR (Yamamoto et al., 2000). The identification of *Clavibacter michiganensis* subsp. *michiganensis* strain was done using specifics primers CMM5F and CMM6R (Dreier, Bermpohl, & Eichenlaub, 1995). All of them were also characterized based on Gram stain, colony morphology in NAD and YDC media (Schaad, Jones, & Chun, 2001) and their pathogenicity was tested. They were stored in nutrient broth medium (OXOID, UK) mixed with glycerol (15% v/v), in an ultra-freezer (-20±2 °C), at the collection of the Department of Plant Protection of the Universidad de la República, Uruguay. The stock cultures were grown and subsequently subcultured for the *in vitro* trial on nutrient agar (OXOID, UK) supplemented with Bacto-Dextrose (Difco Laboratories, USA) at 25±2 °C for 48 h. Then, suspensions of bacterial cells ($1x10^8$ u.f.c. mL^{-1}) were prepared in sterile saline solution (0.85%).

The antimicrobial activity of the hydroalcoholic extracts (EH-P and EH-GP) with three two-fold aqueous dilutions of each one, were tested *in vitro* against the above listed tomato plant pathogenic bacteria by the agar-well diffusion method (Balouiri, Sadiki, & Ibnsouda, 2016). Hence, four treatments per type of propolis were performed: D1 (100%), D2 (50%), D3 (25%), D4 (12.5%). Also, a solvent control (HA) and a control without aggregates (C) were incorporated in all replicates. The assay consisted of Petri dishes containing 18 mL of nutrient dextrose agar mixed with 2 mL of bacterial suspension. Once the agar was solidified, six equidistant wells (6 mm in diameter) were punched per plate using a sterile stainless steel borer. The wells were then randomly filled with 10 **µ**l of EH-P or EH-GP treatments and controls. Each plate was replicated five times. All dishes were incubated in the dark at 25±2 °C and the diameters of the growth inhibition zones around the holes were measured after 24, 48, and 72 h of incubation.

The data were evaluated using analysis of variance (ANOVA) under a randomized complete block design with blocks consisting of the plates. Five replicates of each plate were done. The diameters of the growth inhibition zone were transformed with the square root transformation to meet ANOVA assumptions. When a significant F-value was detected, the diameter means were compared with Fishers Least Significant Difference (LSD) test ($p < 0.05$). The trial was repeated twice.

The results of the chemical characterization of P raw sample were the following: 4.59% moisture, 31.16% waxes, and 2.42% ashes. On the other hand, GP presented a mean value of 7.78% moisture,

50.79% waxes and 3.69% ashes. The amount of total polyphenol content in the EH-P was 3.83 mg eq galic acid mL^{-1} while EH-GP showed 0.16 mg eq galic acid mL^{-1}. The results of the dry extract determination were 78.70 mg mL^{-1} (7.87%) for EH-P and 15.0 mg mL^{-1} (1.50%) for EH-GP. We also observed that P exhibited a physical resinous appearance while GP presented a waxy and sandy aspect.

It was interesting to notice that P showed consistent characteristics with data recorded from other Argentinean propolis samples that were also in agreement with the requirements of the legislation (Bedascarrasbure et al., 2006; Cibanal et al., 2019). Instead, since the chemical composition of stingless bee products are less studied (Silva Araújo et al., 2016), geopropolis comparisons were limited. Geopropolis showed considerable higher values of moisture, ashes, and waxes, and a lower content of total polyphenol than the reported by Brodkiewicz et al. (2018) for *S. jujuyensis* geopropolis collected in Tucumán Province (Argentina). Furthermore, our results were also different from those obtained by Silva Araújo et al. (2016) for four types of geopropolis from Brazil. This can be explained by the fact that this variety of propolis is an extraordinarily variable material. Samples from different geographical regions and bee genera may possess diverse characteristics, as its composition depends on stingless bee's collecting behaviour and the vegetation surrounding the hives (Lavinas et al., 2019). With regard to the content of GP waxes, this considerably high value can be in part attributed to the harvest method, as GP was collected by scraping, and to the resin supply near the apiary. According to Cibanal et al. (2019), the use of plastic collectors unlike what happens with scraping, avoids accidentally adding other materials such as wax to propolis during the harvest.

The analyses of the polyphenol profile by HPLC-DAD (Figure 1) confirmed the presence of caffeic acid, quercetin, 1,5,7-trihydroxy-flavanone, apigenin, pinobanksin, chrysin, pinocembrin, and galangin in both samples, as described by other authors (Kasote et al., 2017; Lavinas et al., 2019). Nevertheless, this study revealed distinctive profiles of the samples as was expected due to the different nature of the products. Propolis sample was constituted mainly of flavonoids and their derivatives such as pinobanksin, chrysin, and pinocembrin. In contrast, GP sample presented in its composition ferulic acid and coumaric acid. Flavonoids, such as pinocembrin, galangin and pinobanksin are considered the principal responsible for the antimicrobial action of propolis mainly by blocking the cell division. In addition, caffeic acid and ferulic acid have also been cited as bactericidal and bacteriostatic components towards some gram-positive and gram-negative bacteria (Ghisalberti, 1979; İnşaatçı & Turan, 2018). All of these observations helped to explain the outcomes of the antimicrobial trial as the biological activity depends mainly on the types and quantity of phenolic compounds (Popova, Silici, Kaftanoglu, & Bankova, 2005).

The *in vitro* assay showed that the extracts differed in their antimicrobial activity. In addition, the tested bacteria displayed significant differences ($p<0.01$) in the sensitivity to each product (Table 1). EH-P treatments had an inhibitory effect over four of the five tested bacteria while EH-GP dilutions were only effective against *C. michiganensis* subsp. *michiganensis* and to a lesser extent. Among the susceptible bacteria to EH-P treatments, the sequence of sensitivity was: *C. michiganensis* subsp. *michiganensis* > *X. gardneri* > *X. vesicatoria* > *P. corrugata*. The extracts' antimicrobial activity was dose-dependent being the most concentrated treatments (78.7 mg mL^{-1} of propolis and 15.0 mg mL^{-1} of geopropolis) the most effective (LSD $p<0.05$, Table 1). The D1 of EH-P was the most effective treatment: it inhibited to a greater extent the growth of all the tomato plant pathogenic bacteria except for *P. mediterranea,* and its action remained almost constant up to 72 h of incubation. Neither of the control treatments (C and HA) showed an inhibitory effect (data not shown). Thus, this confirmed that the antimicrobial activity was due to the presence of bioactive compounds in the EH-P and EH-G, and not because of the alcohol present as a solvent.

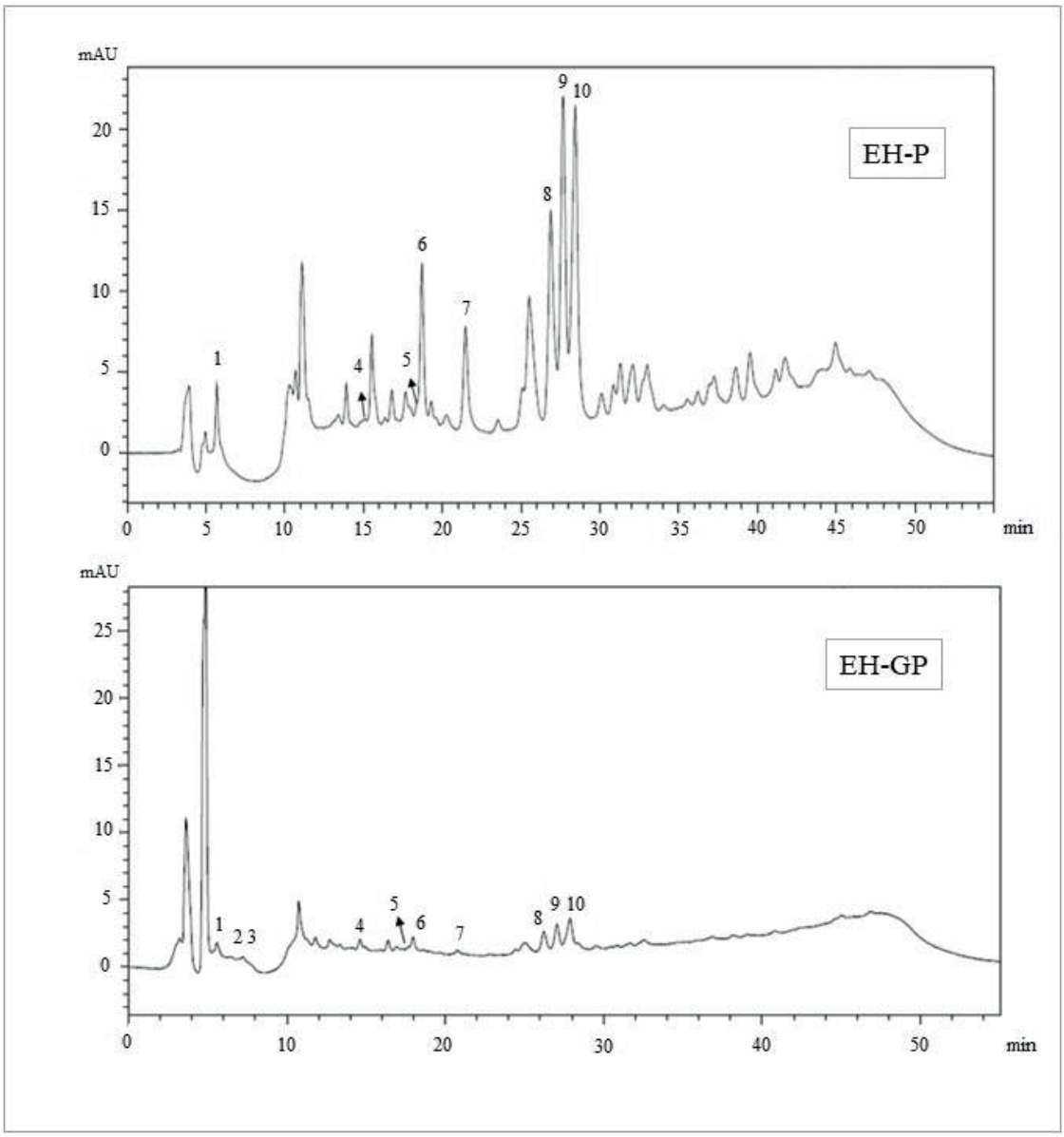

Figure 1. HPLC-DAD chromatograms of the hydroalcoholic extracts (100 mg mL^{-1}) of propolis (EH-P) collected in Río Negro Province (Argentine) and geopropolis (EH-GP) obtained from Formosa Province (Argentine). Main components: 1. Caffeic Acid; 2. Coumaric Acid; 3. Ferulic Acid; 4. Quercetin; 5. 1,5,7-Trihydroxy-flavanone; 6. Apigenin; 7. Pinobanksin; 8. Chrysin; 9. Pinocembrin; 10. Galangin.

The differences in the antimicrobial activity between EH-P and EH-GP could be explained by the quantitative and qualitative differences in their chemical composition, mainly in the total polyphenol content and in the amount of dry extract (Figure 1). In this sense, the results of these trials were in accordance with Popova et al. (2005), who evidenced the importance of the amount of phenolic compounds for the antimicrobial activity of propolis. With regard to the dry extract content, it is important to note that the use of hydroalcohol as a solvent extracts more bioactive compounds and less wax from propolis samples (Sforcin & Bankova, 2011). This may have determined the low content of dry

extract in EH-GP, as the raw sample was composed mainly by waxes. The choice of hydroalcohol is also supported by the studies of Silva Frozza et al. (2013), who indicated that this extractant is able to solubilise phenols and other bioactive compounds in considerable quantities, and that it is less toxic than other solvents.

Table 1
Mean diameters of the growth inhibition zones around the holes (mm) of propolis and geopropolis hydroalcoholic extracts (EH-P and EH-GP) dilutions (D1, D2, D3 and D4), after 24, 48, and 72 h of incubation tested *in vitro* by the agar-well diffusion method. The two controls used are no expressed, since there was no inhibition

Tomato phytopathogenic bacteria	EH-P											
	24h				48h				72h			
	D1	D2	D3	D4	D1	D2	D3	D4	D1	D2	D3	D4
	78.7*	39.3*	19.6*	9.8*	78.7*	39.3*	19.6*	9.8*	78.7*	39.3*	19.6*	9.8*
Clavibacter michiganensis subsp. *michiganensis*	15.5 a	11.2 a	9.5 a	9.0 a	15.2 a	9.7 b	9.7 b	8.0 b	15.0 a	7.7 bc	9.5 b	6.5 c
Xanthomonas gardneri	4.0 a	2.7 ab	2.0 b	0.5 c	4.0 a	2.7 ab	0.5 b	0.5 c	2.5 a	1.2 b	1.2 b	-
Xanthomonas vesicatoria	1.5 a	0.5 b	0.2 b	-	1.0	-	-	-	1.0	-	-	-
Pseudomonas corrugata	0.5	-	-	-	0.5	-	-	-	0.5	-	-	-
Pseudomonas mediterranea	-	-	-	-	-	-	-	-	-	-	-	-
Tomato phytopathogenic bacteria	**EH-GP**											
	24h				48h				72h			
	D1	D2	D3	D4	D1	D2	D3	D4	D1	D2	D3	D4
	15	7.5	3.7	1.8	15	7.5	3.7	1.8	15	7.5	3.7	1.8
Clavibacter michiganensis subsp. *michiganensis*	1.5 a	1 ab	0.7 ab	0.5 b	1.0	-	-	-	0.7	-	-	-
Xanthomonas gardneri	-	-	-	-	-	-	-	-	-	-	-	-
Xanthomonas vesicatoria	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas corrugata	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas mediterranea	-	-	-	-	-	-	-	-	-	-	-	-

Means with different letters in the same line for each time differ significantly at $p \leq 0.05$ according to Fisher´s LSD test.
-: Not detected.
*: Dilutions dry extract (mg mL^{-1}).

On the other hand, the differences found between the sensitivity of the tested bacteria to the extracts, could be explained in part because propolis and geopropolis are highly active against Gram-positive bacteria (*C. michiganensis* subsp. *michiganensis)*, and show limited activity against the Gram-negative ones (*P. corrugata*, *P. mediterranea*, *X. gardneri, X. vesicatoria*) (Mendes Araújo et al., 2015). Also, according to these authors, Gram-negative bacteria have a more complex cell wall and higher lipid content, which may explain their resistance to propolis extracts. In another sense, Tosi, Donini, Romagnoli, and Bruni (1996) observed that propolis solutions were inactive against phytopathogenic bacteria of the genera *Pseudomonas* sp. This helped to explain that the isolates of *Pseudomonas* sp. used did not respond to any treatment. Further assays are needed to determine if more concentrated extracts have a better performance against these bacteria.

The use of propolis and geopropolis in agriculture is still poorly described in literature. Basim, Hüseyin and Özcan (2006) first reported the antimicrobial activity of Turkish propolis against 13 different species of plant pathogenic bacteria including *C. michiganensis* subsp. *michiganensis, P. corrugata, Xanthomonas campestris,* and *X. vesicatoria.* Like the results from this work, they observed that honey bee propolis was highly active against Gram-positive bacteria, and showed limited action against the Gram-negative ones. The inhibition zone diameters reported by these authors, are comparable with EH-P treatments against *C. michiganensis* subsp. *michiganensis*. Further, Cibanal et al. (2019) found *in vitro* antimicrobial activity of different Argentinean propolis against plant pathogenic fungi. In view of the economic importance of tomato, the possibility of using products with a lower risk to human health and the environment is very promising.

In conclusion, the polyphenol content and the HPLC-DAD profile of the hydroalcoholic extracts showed differences in chemical composition that were related to the *in vitro* bioactive effects. Propolis from *A. mellifera* showed antimicrobial effect against tomato plant pathogenic bacteria and especially on *C. michiganensis* subsp. *michiganensis*. In contrast, activity of geopropolis from *S. jujuyensis* was limited and further studies are needed to assay different types of geopropolis. These results are a contribution to the characterization of bee bioactive products, specifically to propolis and geopropolis. This study indicates the likelihood of using propolis as a non-conventional strategy to control tomato bacterial diseases and encourages to continue with research on this subject.

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