**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Evaluation of the residues from the ethanolic extraction of organic propolis as
a source of biological compounds**

Natália Yumi Ikeda

Thesis presented to obtain the degree of Doctor in
Science. Area: Food Science and Technology

**Piracicaba
2020**

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DEDICATION

**Ao meu amigo e companheiro,
Fabio Henrique Takahashi**

"Keep moving forward"

Walt Disney

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RESUMO

Avaliação dos resíduos obtidos da extração etanólica da própolis orgânica como fonte de compostos biológicos

O extrato etanólico de própolis (EEP) é a forma mais eficiente de extrair os compostos biológicos da própolis. Entretanto, a cadeia produtiva do EEP produz grande quantidade de resíduos. Esse resíduo é composto por diversas substâncias como cera, resinas e gomas, e ainda pode conter alguns compostos biológicos. Entretanto, esse resíduo não pode ser reutilizado para o consumo humano, mas poderia ser aproveitado na alimentação animal. Este estudo investigou duas formas diferentes de utilizar os resíduos da extração etanólica da própolis orgânica, a extração de óleos essenciais (OEs) e de frações de ácidos fenólicos. Os OEs foram extraídos por hidrodestilação e as frações de ácidos fenólicos foram obtidas por extrações com solventes e hidrólise alcalina/ácida. Ambos os compostos foram avaliados em relação a concentração de fenólicos totais, capacidade antioxidante por ABTS, DPPH e FRAP e atividade antibacteriana contra um modelo de bactéria patogênica (*Escherichia coli*) e uma benéfica (*Lactobacillus plantarum*). Todos os parâmetros foram comparados com o extrato etanólico de própolis (EEP). Este estudo teve como objetivo investigar os resíduos da própolis como fonte de compostos biológicos que poderiam ser adicionados na alimentação animal como uma alternativa aos antimicrobianos sintéticos.

Palavras-chave: Ácidos fenólicos, *E. coli*, *Lactobacillus*, Óleo essencial, Resíduo

ABSTRACT

Evaluation of the residues from the ethanolic extraction of organic propolis as a source of biological compounds

Ethanolic extract of propolis (EEP) is one of the most effective ways to extract the active compounds of propolis resin. However, EEP's processing chain generates a great amount of residues. This residue is composed of several substances such as wax, resins and gums, and also could still contains some bioactive compounds. However, it cannot be reused for human consumption, but it could be applied in animal feed. This study investigated two different forms to use the residues from ethanolic extraction of organic propolis, the extraction of essential oils (EOs) and phenolic acids fractions. EOs were extracted through hydrodistillation and phenolic acids fractions were obtained through solvent extractions and alkaline/acid hydrolysis methods. Both compounds were evaluated for total phenolic content, antioxidant capacity in terms of ABTS, DPPH and FRAP, and antibacterial activity against a pathogenic (*Escherichia coli*) and a beneficial (*Lactobacillus plantarum*) bacterium model. All parameters were compared with ethanolic extract of propolis (EEP). This study aimed to investigate propolis residues as a source of biological compounds that could be added in animal feed as an alternative to synthetic antimicrobials.

Keywords: *E. coli*, Essential oil, *Lactobacillus*, Phenolic acids, Propolis residue

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1. INTRODUCTION

Propolis is an apicultural term for the resins harvested from various plant species by honey bees and it is considered a promising source of compounds for the discovery of new pharmaceuticals (DE ALBUQUERQUE et al., 2008; KAŠKONIENE et al., 2014; RIOS et al., 2014; BITTENCOURT et al., 2015; DARENDELIOGLU et al., 2016; DO AMARAL DUARTE et al., 2017). Since propolis compounds are generally recognized as safe (GRAS) substances, they were frequently used as food ingredients and/or food additives (KAŠKONIENE et al., 2014). It is mainly composed by resin (60%), being the remaining compounds comprised by waxes, essential oils, vitamins and microelements (BANKOVA; POPOVA; TRUSHEVA, 2014; REIS et al., 2017). As such compounds have a plant origin, the composition of the plant source determines the chemical composition of propolis (DE ALBURQUERQUE et al., 2008; MIGUEL; ANTUNES, 2011; BANKOVA; POPOVA; TRUSHEVA, 2014; FERNANDES et al., 2015).

Various biological properties have been ascribed to propolis including antimicrobial, antioxidant, antitumor, anti-inflammatory, anti-ulcer, anesthetic and anti-HIV activities (ITO et al., 2001; DE CASTRO ISHIDA et al., 2011; ZHU et al., 2011a, 2011b; KRÓL et al., 2013; SULEMAN et al., 2015). The bioactivity of propolis is believed to be a result of its complex chemical composition, which harbors a wide range of bioactive compounds (FERNANDES et al., 2015; KAMATOU et al., 2019). The complex form of propolis enables the utilization of different extraction solvents such as ethanol, methanol and water. However, propolis ethanolic extract is the most produced once it consists in a richest phenolic acids and flavonoids solution (MIGUEL; ANTUNES, 2011; DARENDELIOGLU et al., 2016).

After the ethanolic extraction of crude propolis, the ethanolic solution have a high market value being used as a final dosage form or occasionally incorporated into foods, beverages, medicines or cosmetics. However, the resinous part constitutes the waste material of propolis, which represents almost 80-94% of propolis used to obtain the extract, being composed of several substances such as wax, resins and gums (HEIMBACH et al., 2014; HEIMBACH et al., 2016; DO AMARAL DUARTE et al., 2017; DE FRANCISCO et al., 2018). Nevertheless, it cannot be use directly on food industry due to its resinous composition (HEIMBACH et al., 2014; REIS et al., 2017; DE FRANCISCO et al., 2018). Therefore, the subjection of this material to a second extraction or other process could originate a rich and interesting ingredient that can be used by different industries (DE

FRANCISCO et al., 2018). This material could provide some active compounds with potential application in animal nutrition (REIS et al., 2017; SANTOS et al., 2013).

2. LITERATURE REVIEW

2.1. Propolis

Bees have the ability to collect plant compounds that can protect the beehive. Propolis (bee glue) is one of the protective products resulting from this behavior (BANKOVA; POPOVA; TRUSHEVA, 2014; FERNANDES et al., 2015). The compounds present in the propolis resin have three origins: substances actively secreted by plants and substances exuded from wounds in plants (lipophilic materials on leaves and leaf buds, resins, mucilage, gums, among others) collected by bees, secreted substances from bee metabolism, and materials introduced during propolis elaboration (MIGUEL; ANTUNES, 2011; GALEOTTI et al., 2018; KAMATOU et al., 2019). It is nearly constituted by resin (60%), being the remaining composition comprised by waxes, essential oils, vitamins and microelements (REIS et al., 2017). As such compounds have a plant origin, the composition of the plant source determines the chemical composition of propolis (DE ALBURQUERQUE et al., 2008; MIGUEL; ANTUNES, 2011; BANKOVA; POPOVA; TRUSHEVA, 2014; FERNANDES et al., 2015). Although propolis is obviously an animal product, most of the components responsible for biological activities are derived from plants (GALEOTTI et al., 2018).

Bees use the mechanical properties of this resinous substance to block holes and cracks, repair combs, strength the thin borders of the comb, seal the openings in the hive to avoid the entrance of intruders and maintain a constant inner temperature (BANKOVA; POPOVA; TRUSHEVA, 2014; GALEOTTI et al., 2018; KAMATOU et al., 2019). Propolis also consolidates structural components and varnish inside the cells with disinfecting purposes and prevent vibrations (MENDIZABAL, 2005; OLIVIERA et al., 2010; RIOS et al., 2014). On the other hand, bee glue restrains the putrefaction of “embalmed” intruders that were killed in the hive and are too large to be carried out, contributing to the attainment of an internal aseptic environment and the protection of the hive from widespread bacterial infection (BANKOVA; POPOVA; TRUSHEVA, 2014; FERREIRA et al., 2017; GALEOTTI et al., 2018; KAMATOU et al., 2019).

The composition of propolis varies from hive to hive, from district to district, and depends on the time of collection, seasonality, illumination, altitude, collector type, and food availability and activity developed during propolis exploitation. Honeybees are opportunists, gathering

what they need from available sources. Thus, the chemical composition of propolis varies considerably from region to region, along with the vegetation (ORYAN; ALEMZADEH; MOSHIRI, 2018).

The chemical composition of propolis is very complex and high variable according to its botanical and phytogeographical origin (BANKOVA, 2009). As a consequence, the different propolis types are characterized by distinct chemical profiles, according to their plant origin, with the presence of specific compound types such as polyphenols, terpenoids, prenylated acetophenones and isoflavonoids (VELIKOVA et al., 2000; KUMAZAWA, HAMASAKA, NAKAYAMA, 2004; MELLIU, STRATIS, CHINOU, 2007; POPOVA et al., 2017).

2.1.1. Biological properties of propolis

Various biological properties have been ascribed to propolis, including antimicrobial, antioxidant, antitumor, anti-inflammatory, anti-ulcer, anesthetic and anti-HIV activities (ITO et al., 2001; DE CASTRO ISHIDA et al., 2011; ZHU et al., 2011a, 2011b; KRÓL et al., 2013; SULEMAN et al., 2015). The bioactivity of propolis results from its complex chemical composition, which harbors a wide range of bioactive compounds (FERNANDES et al., 2015; KAMATOU et al., 2019). These effects are exerted by the numerous chemical compounds identified in the volatile and non-volatile fractions of propolis of different botanical and geographical origin (BANKOVA; POPOVA; TRUSHEVA, 2014; KAŠKONIENE et al., 2014; DARENDELIOGLU et al., 2016; GALEOTTI et al., 2018; KAMATOU et al., 2019). The most important biologically active compounds are polyphenols, including flavonoids, phenolic acids and their esters (PELLATI; PRENCIPE; BENVENUTI, 2013; GALEOTTI et al., 2018).

Propolis is an apicultural term for the resins harvested from various plant species by honeybees and it is considered a promising source of pharmaceutical compounds. In the last decades, several works dealing with propolis composition and biological properties have been published (DE ALBUQUERQUE et al., 2008; KAŠKONIENE et al., 2014; RIOS et al., 2014; BITTENCOURT et al., 2015; DARENDELIOGLU et al., 2016; DO AMARAL DUARTE et al., 2017). Some studies have also highlighted the potential of propolis as a dietary supplement and food preservative due to its biological properties (MU et al., 2006; CANDIR et al., 2009; YANG et al., 2010). Since propolis compounds are generally recognized as safe (GRAS) substances, they are frequently used as food ingredients and/or food additives (KAŠKONIENE et al.,

2014). However, propolis is barely soluble in water and cannot be used as a raw material, so it must be purified by extraction with solvents to remove the inert material and preserve the polyphenolic fraction (KARLA et al., 2017; POPOVA et al., 2017). Flavonoids and phenolic acids are considered the responsible of most biological effects of propolis (MIGUEL; ANTUNES, 2011; KARLA et al., 2017).

The polarity of the solvent and the propolis composition are two major factors able to influence the quality of propolis extracts (GALEOTTI et al., 2018). Propolis extracts are commonly obtained through conventional techniques such as ethanolic or aqueous extraction. In fact, propolis is added to commercial and supplemental health care products in form of extracts obtained by soaking crushed propolis in organic solvent or water (PIETTA et al., 2002; KARLA et al., 2017; GALEOTTI et al., 2018). The ethanolic extract consists in a fraction rich in phenolic acids and flavonoids (DARENDELIOGLU et al., 2016; POPOVA et al., 2017).

The distinct chemistry of propolis from diverse origins does not mean dissimilar properties. Bees can find diverse components with biological properties in the flora that surrounds their beehives (MIGUEL; ANTUNES, 2011). The composition of propolis is very complex and varies depending on the phytogeography diversity of the collection area and the season. Because of the variability of plant sources, the antibacterial compounds in bee glue are different in distinct geographic regions. For example, flavonoids and cinnamic acid derivatives are commonly detected in European samples, whereas diterpenic acids and prenylated coumaric acids are highly present in Brazilian samples (POPOVA et al., 2017). Seidel et al. (2008) compared the antibacterial activity of propolis from different geographical origins, including countries from tropical, subtropical, and temperate zones, and they found that propolis extracts were mostly active against Gram-positive bacteria. In particular, propolis from wet-tropical, rainforest-type climate had the highest antibacterial activity. Ethanolic extracts of propolis were also more effective against Gram-positive bacteria than against Gram-negative bacteria (MIGUEL; ANTUNES, 2011). In fact, they seemed to stop the growth of Gram-negative bacteria only at high concentration of propolis. This could be explained as one of the most aggressive diseases that affect the hive is caused by the Gram-positive bacterium *Paenibacillus larvae* (FERREIRA et al., 2017).

The antimicrobial activity of propolis is due to its active compounds such as aromatic compounds and flavonoids (PAROLIA et al., 2010; ANJUM et al., 2018). According to

Mirzoeva et al., (1997), propolis had bacteriostatic action on Gram-positive and some Gram-negative bacteria. Propolis apparently modifies the bioenergetics status of bacterial membrane and inhibits bacterial motility (HEIMBACH et al., 2016). Moreover, propolis acts as a bactericidal agent, stopping division of bacterial cell, destroying the cell wall and stopping the protein synthesis (LOTFY, 2006; PAROLIA et al., 2010; MACHADO et al., 2016; ANJUM et al., 2018). Propolis has a significant effect against *Enterococcus* ssp. and *Staphylococcus aureus* (AL-WAILI et al., 2012; KUROPATNICKI et al., 2013).

Oxidation is an essential process to all living organisms as it produces energy necessary for their proper functioning. Uncontrolled oxidation generates excessive amounts of free radicals, which results in lethal cell changes such as the oxidation of lipids, proteins, and carbohydrates. Hence, the search of antioxidant substances which neutralize and prevent the action of free radicals by avoiding the formation of radicals, scavenging them, or by promoting their decomposition have gained more attention (YOUNG & WOODSIDE, 2001; CASTRO et al., 2014). Both natural and synthetic antioxidants have been used in medicine and in the food industry. However, synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are suspected to have a noxious effect on the human body, being extensively examined their potential toxicity (OLSZOWY; DAWIDOWICZ, 2016; WANG et al., 2017). Thus, natural antioxidants have attracted increasing interest because they may be safer than the synthetics (WOLFE et al., 2003; MANACH et al., 2004; SOARES et al., 2008; AMORATI; FOTI; VALGIMIGLI, 2013; WANG et al., 2017). Phenolic compounds have been extensively studied as antioxidants present in natural products that are able to prevent the formation of free radicals (YOUNG & WOODSIDE, 2001; PERRON & BRUMAGHIM, 2009; CASTRO et al., 2014). The antioxidant activity of propolis is due to phenolic compounds, which donate hydrogen ions to free radicals to protect cells from oxidative reactions. Propolis had the capability to remove free radicals, which are the primary cause of lipids, nucleic acids and proteins oxidation (ANJUM et al., 2018).

2.1.2. Propolis residues

Sustainable approaches have become an essential challenge for different industries, aiming to minimize the environmental impact of their residues. Generally, tons of residues are created and discarded during the manufacturing process leading to environmental problems.

Thus, the valorization of food residues is an economic, social and scientific concern. In the case of propolis, it is expected that the propolis market grow a rate of 3.50% until 2021, increasing the production from 2300 t in 2015 to 2900 t in 2021 (DE FRANCISCO et al., 2018). After the ethanolic extraction of crude propolis, the ethanolic solution have a high market value being used as a final dosage form or occasionally incorporated into foods, beverages, medicines or cosmetics. However, the resinous part constitutes the waste material of propolis, which represents almost 80-94% of propolis used to obtain the extract, being composed of several substances such as wax, resins and gums (HEIMBACH et al., 2014; HEIMBACH et al., 2016; DO AMARAL DUARTE et al., 2017; DE FRANCISCO et al., 2018).

2.2. Essential oils

In particular, the volatile composition of propolis can differ significantly depending on the sample, despite of the presence of a similar phenolic composition (BANKOVA; POPOVA; TRUSHEVA, 2014). The main volatile compounds in propolis are terpenoids (MELLIU et al., 2007; POPOVA et al., 2017), in which sesquiterpenoid alcohols and hydrocarbons predominate, accompanied by some monoterpenes, mainly alcohols (BANKOVA; POPOVA; TRUSHEVA, 2014; MIGLIORI et al., 2016). Volatile compounds like those found in essential oils are in low concentrations in propolis, but they are responsible of propolis pleasant aroma and different biological activities. Distillation-extraction or hydrodistillation have been widely used to obtain propolis volatiles (BANKOVA; POPOVA; TRUSHEVA, 2014; SENA-LOPES et al., 2018).

A large number of biological activities have been reported for essential oils (EOs) such as antimicrobial, antiviral, antimycotic, antiparasitic, insecticidal, antidiabetic, antioxidant, and anticancer (EDRIS, 2007; REICHLING et al.; 2009). The biological activities are related with EOs bioactive compounds, as well as the functional groups and structure arrangement from these molecules (BURT, 2004; LOPEZ-ROMERO et al., 2015).

2.2.1. Antimicrobial activity

Due to the accepted safe status of some natural products, the interest in antimicrobials derived from nature has increased. Plants are used for thousands of years in traditional

medicine to treat infectious and they continue to play an important role in the discovery of new molecules. Within plant secondary metabolites, EOs contain diverse bioactive compounds with chemical and structural variance that make them versatile in terms of functions. Thus, EOs represent a potential source of novel antimicrobial agents that have attracted special attention (LOPEZ-ROMERO et al., 2015).

EOs exhibit antimicrobial activity against a large number of Gram-negative and Gram-positive bacteria. It has been observed that the mode of action of EOs is based on their ability to disrupt cell wall and cytoplasmic membrane, leading to lysis and leakage of intracellular compounds (NEWMAN & CRAGG, 2012; LOPEZ-ROMERO et al., 2015). It is possible to conclude that membrane/cell wall permeability can be related to alterations on their physicochemical properties (hydrophobicity and charge). Sikkema et al. (2010) showed that the lipophilic character of monoterpenoid components allows them to partition in the aqueous phase into the membrane structures, increasing membrane fluidity and permeability, disturbance of membrane proteins, inhibition of respiration, and alteration of ion transport process. Helander et al. (1998) described the effect of various EOs components on the outer membrane permeability in Gram-negative bacteria, evidencing that monoterpene capture is determined by the permeability of the target microorganism.

2.2.2. Antioxidant properties

The search for natural antioxidants with the virtue of being nontoxic has given rise to a large number of studies on the antioxidant potential of EOs. This is particularly relevant because most common synthetic antioxidants (such as butylated hydroxyl anisole (BHA) or butylated hydroxyl toluene (BHT)) are suspected to be potentially harmful to human health (AMORATI; FOTI; VALGIMIGLI, 2013).

The mechanism of antioxidant activity expressed by EOs is related to their composition. Despite the observed large chemical diversity, the main components of common EOs can be classified in two structural families with respect to hydrocarbon skeleton: terpenoids, formed by the combination of two (monoterpene), three (sesquiterpene), or four (diterpene) isoprene units, and phenylpropanoids. Both terpenoid and phenylpropanoid families comprise phenolic compounds, sometimes accounted among principal components of several EOs (AMORATI; FOTI; VALGIMIGLI, 2013; LOPEZ-ROMERO et al., 2015). The overall

performance as antioxidant is, in fact, the result of the complex interaction among components and the oxidizable material to be protected. Synergistic or antagonistic behaviors are thus expected, depending on the EO composition and experimental conditions (AMORATI; FOTI; VALGIMIGLI, 2013).

Despite the reduced number of studies on the essential oil of propolis (EOP) from different world regions, a wider variability in chemical composition has been found in EOP than among non-volatile constituents (FERNANDES et al., 2015).

2.3. Free and bound phenolic acids

Phenolic acids and their derivatives are widely distributed in plants, being most of them essential metabolites. Phenolic compounds are considered secondary metabolites synthesized by plants during a normal development or in response to stress conditions such as infection, wounding and UV radiation, among others (KRYGIER; SOSULSKI; HOGGE, 1982; HARBONE, 1982; NACZK; SHAHIDI, 2004; .ZHANG; TSAO, 2016). Plant phenolics include simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins. Plant phenolics may act as phytoalexins, antifeedants, attractants for pollinators, contributors to the plant pigmentation, antioxidants and protective agents against UV light, among others (NACZK; SHAHIDI, 2004).

The antioxidant and anti-inflammatory activities of polyphenols, as well as other biological functions, have been largely attributed to their particular chemical structures. The aromatic feature and highly conjugated system with multiple hydroxyl groups make these compounds good electron or hydrogen atom donors, neutralizing free radicals and other reactive oxygen species (ROS) (BONOLI et al., 2004; ZHANG; TSAO, 2016).

Extraction of phenolic compounds in plant materials is influenced by their chemical nature, the extraction method employed, sample particle size, storage time and conditions, as well as presence of interfering substances (NACZK; SHAHIDI, 2004). The chemical nature of plant phenolics vary from simple to highly polymerized substances that include varying proportions of phenolic acids, phenylpropanoids, anthocyanins and tannins, among others (NACZK; SHAHIDI, 2004). They also exist as complexes with carbohydrates, proteins and other plant components, and some high-molecular-weight phenolic and their complexes may be quite insoluble. Therefore, phenolic extracts of plant materials are always a mixture

of different classes of phenolic that are soluble in the solvent system used (NACZK; SHAHIDI, 2004).

Solubility of phenolic compounds depend on the type of solvent (polarity) used, degree of polymerization, as well as interaction with other constituents and formation of insoluble complexes (NACZK; SHAHIDI, 2004). Thus, the total phenolic content of plant material has often been underestimated, because the content of bound phenolic compounds, usually found in significant quantities, was not determined. Methanol, ethanol, acetone, water, ethyl acetate and, to a lesser extent, propanol, dimethylformamide, and their combinations are frequently used for the extraction of phenolics (BONOLI et al., 2004; NACZK; SHAHIDI, 2004). However, none of these solvents achieved a suitable extraction of all phenolics or a specific class of phenolic substances in plant materials.

Free phenolic compounds are solvent extractable in contrast to bound phenolic compounds, which are covalently bound to the plant matrix and cannot be extracted into water or aqueous/organic solvents mixtures (SU et al., 2014). Bound phenolic compounds are covalently conjugated to cellulose, pectin and polysaccharides through ester bonds, being difficult to hydrolyze (BONOLI et al., 2004; KHODDAMI; WILKES; ROBERTS, 2013; SU et al., 2014). Additional steps may be required for the removal of unwanted phenolic and non-phenolic substances such as waxes, fats, terpenes and chlorophylls (NACZK; SHAHIDI, 2004). Alkaline, acid or enzymatic hydrolysis methods can be used to release bound phenolic compound (BONOLI et al., 2004; KHODDAMI; WILKES; ROBERTS, 2013; SU et al., 2014).

Alkaline pressure-hydrolysis was one of the first methodologies used to detect compounds attached to the high molecular weight fraction. Saponification or alkaline hydrolysis is frequently used to release covalently bound phenolic compounds. Acid conditions have also been used on cereals, fruits, vegetables and beverages to release phenolic compounds covalent linked to other structures (MONENTE et al., 2015). Barbeau and Kinsella (2001) reported that a high ionic strength medium with NaCl decreases the bindings of chlorogenic acids to protein fractions. Another study showed that higher concentrations of phenolic acids are obtained after the addition of NaCl, confirming that NaCl breaks the ionic bindings between phenolic compounds and proteins (MONENTE et al., 2015). High ionic strength solutions also break non-covalent bonds between melanoidins and low molecular weight compounds such as phenolics (MONENTE et al., 2015).

Free or simple conjugates of polyphenols are absorbed in the upper gastrointestinal tract, but their bioavailability is very low compared to the vitamins. Phenolic compounds are mainly metabolized by the gut microbiota in the colon before their delivery to tissues or organs (CLIFFORD, 2004; ZHANG; TSAO, 2016; OBOH et al., 2017). In particular, bound phenols may resist digestion and reach the colon intact, where they are released by the action of microbial beta-glucosidase and exert their biological effects (OBOH et al., 2017). Phenolics can therefore positively affect local inflammatory status, or indirectly act as prebiotics to promote the growth of probiotics, leading to improved gut health (ZHANG; TSAO, 2016; OBOH et al., 2017).

The ability of dietary polyphenols to reduce inflammation is considered, acting as antioxidants, interfering with the oxidative stress signaling, and suppressing the pro-inflammatory signaling transductions (MANACH et al., 2005; SOARES et al., 2008; ZHANG; TSAO, 2016). Phenolic compounds are recognized as direct antioxidants, but they may also exhibit indirect antioxidant effects through the induction of endogenous protective enzymes, and beneficial regulatory effects on signaling pathways (STEVENSON AND HURST, 2007). In the gut, phenolic compounds may selectively suppress or stimulate the growth of some components of intestinal microbiota, influencing the bacterial population dynamics (TZOUNIS et al., 2008; CUEVA et al., 2010).

Unabsorbed dietary polyphenols and their metabolites can behave as activators or inhibitors of bacterial growth depending on their chemical structure (substitutions in the phenolic ring) and concentration (VIVA et al., 1997; REGUANT et al., 2000). These metabolites selectively inhibit pathogen growth and stimulate the growth of commensal bacteria, including also some recognized probiotics (LEE et al., 2006; LARROSA et al., 2009; CUEVA et al., 2010), thus influencing the microbiota composition (LAPARRA & SANZ, 2010). Studies conducted in humans, rats, pigs and chickens have revealed that the administration of polyphenols from plant sources produces an increase in the growth of beneficial bacteria such as *Lactobacillus* and a decrease in *Enterobacteriaceae*, *Clostridium* and *Bacteroides*, among other bacterial groups (HARA, 1997; DOLARA et al., 2005; MOLAN et al., 2010; VIVEROS et al., 2011). Therefore, polyphenols appear to have potential to confer health benefits via modulation of the gut microbiota and exerting prebiotic-like effects (TZOUNIS et al., 2008; TABASCO et al., 2011). Gut bacteria also have the capacity to metabolize polyphenols and may play a major

role in the production of new phenolic compounds *in situ*, which could have higher bioavailability and biological activity than their parent compounds (REQUENA et al., 2010).

2.4. Antimicrobial alternatives in animal nutrition

Antimicrobial resistance is one of the most serious public health treats nowadays, resulting from the selective pressure exerted by antibiotic use and abuse. During the last decades, the rapid evolution and spread of resistance among clinically important bacterial species have caused that many antimicrobial agents lost their efficacy, which limit the therapeutic options for the treatment of infections (LOPEZ-ROMERO et al., 2015). According to the World Health Organization (WHO) infectious diseases are the second cause of death worldwide. Therefore, it is necessary to develop new alternative compounds to decrease the problem of the microbial resistance.

Antibiotics are extensively used in intensive livestock industries such as swine production as growth promoters, in prophylactic or metaphylactic treatments to prevent diseases and to treat different diseases (BARTON, 2014). Antibiotic resistance in bacteria associated with pigs not only affects pig production, but it also has an impact on human health through the transfer of resistant organisms and associated genes via the food chain and it could also compromise treatment of human infections (BARTON, 2014). Currently, its impact is considerable with treatment failures associated with multidrug-resistant bacteria and it has become a global concern to public health (BALOUIRI et al., 2016). With these concerns, many researchers have focused on the investigation of plant and microbial extracts, essential oils, pure secondary metabolites, and new synthesized molecules as potential antimicrobial alternatives. Plants and other natural sources can provide a huge range of complex and structurally diverse compounds, which could also reduce the bacterial resistance in cooperation with standard antibiotics due to their safety and efficiency (BALOUIRI et al. 2016; WANG et al., 2018).

Enteric and respiratory infections are the most frequent and recurrent diseases in swine industry. Especially, enteric infection is one of the major stressors causing low productivity in farm animals since it suppresses the feed conversion efficiency (KIARIE et al., 2011; YANG et al., 2014). Unlike other type of stressors, infectious stress is not easy to control and often causes long-term economic loss. In an outbreak, the transmission between batches of

animals occurs very fast because most modern animal farms have adopted an intensive rearing system (LEE et al., 2016). Most enteric pathogens infect the animals from drinking water, feeds, or feces from other infected animals. After accessing the animal's gut, enteric pathogens generally disrupt the homeostasis of the epithelial barrier. Some pathogens including *Escherichia coli* induce intestinal damage through osmotic stress causing secretory diarrhea, while others cause diarrhea by up-regulating pro-inflammatory cytokines, producing the so-called inflammatory diarrhea (FAIRBROTHER et al., 2005). Enteric pathogens suppress the feed intake and feed conversion, which cause unnecessary energy loss for activating immune system. Enteric diseases in the swine industry are mainly caused by *E. coli*, porcine epidemic diarrhea virus (PEDV), porcine delta coronavirus (PDCoV), and transmissible gastroenteritis coronavirus (TGEV) (LEE et al., 2016).

E. coli is a gram-negative, enteric species mainly composed by commensal strains. However, some of them are pathogenic, also called enterotoxigenic *E. coli* (ETEC), causing diarrhea accompanied by dehydration, inhibition of the feed conversion and growth performance (YANG et al., 2014). ETEC can also shorten the length of villus and the depth of crypt of small intestine, and inhibit the expression of tight junction of intestinal epithelial cells by loosening the epithelial barrier (YANG et al., 2014). Supplementation of *Lactobacillus plantarum*, *Saccharomyces cerevisiae boulardii*, chitosan or vasoactive intestinal peptide has been reported to alleviate the infection stress caused by ETEC (COLLIER et al., 2011; XIAO et al., 2013; XU et al., 2014; YANG et al., 2014).

The use of propolis and its derivatives during the nutrition of broiler chickens favors the growth of beneficial gut microbiota over pathogenic ones, and consequently improves the digestion and absorption of nutrients (KACÁNIOVA et al., 2012, KITA et al., 2014). However, propolis has active compounds that may decrease the activity of digestive enzymes, such as amylase and maltase (MATSUI et al., 2004; ZHANG et al., 2015), as validated in broilers fed crude propolis (DUARTE et al., 2014) or an ethanolic propolis extract (EYNG et al., 2014; DO AMARAL DUARTE et al., 2017).

Propolis residues could also contain some bioactive compounds with potential to be used in human or animal nutrition (SANTOS et al., 2013; REIS et al., 2017), or they could be applied as structuring agents (ROSSETO et al., 2017). Nevertheless, its resinous composition limits their application in food industry, so they must be subjected to a second extraction process

to produce rich and interesting ingredients that can be used by different industries (DE FRANCISCO et al., 2018).

The application of propolis and its residues on animal performance remains controversial due to their highly variable effects. Furthermore, insufficient literature is available on the use of solid residues from the extraction of propolis, which probably show different effects than those of crude propolis or ethanolic extracts. Many chemical compounds are removed by ethanol extraction of propolis, such as polyphenols. Moreover, propolis residue contains a high level of crude energy (5.718 kcal/kg), due to its high wax quantity (26.8%) (SANTOS et al., 2003). However, propolis residues have low metabolizable energy (941 kcal/kg dry matter) for chickens, because its high wax content is poorly digested (DO AMARAL DUARTE et al., 2017).

Based on the literature, plant extracts may improve animal health through several mechanisms such as direct suppression of the proliferation of pathogens, alteration of gut microbial populations, and enhancement of immune functions (LIU et al., 2018).

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Chapter 1. Chemical composition, antibacterial and antioxidant activity of essential oil of crude organic propolis and its residues

1. ABSTRACT

Ethanol extract of propolis (EEP) is one of the most effective ways to extract the active compounds of propolis resin. However, the EEP's processing chain also generates a great amount of residues. This study aimed to investigate the presence of bioactive compounds in propolis residues. The volatile composition and phenolic content of essential oils of propolis (EOP), moist residue (EOMR) and dry residue (EODR) were determined. The *in vitro* antioxidant capacity (ABTS, DPPH and FRAP) and antibacterial activity against a pathogenic (*E. coli* ATCC 25922) and a beneficial (*L. plantarum* ATCC 8014) bacterium model of these EOs were compared with that of EEP. An EO yield of 1.13% for EOP, 0.12% for EOMR and 0.16% for EODR was obtained after organic propolis extraction. The major compounds present in the EOs were α -pinene, β -pinene, and limonene. EOs were not able to scavenge stable ABTS and DPPH radicals, but the FRAP assay indicated that they were capable to chelate transition metals. EOs showed a selective antibacterial activity against pathogenic bacteria, which could benefit the gut microbiota.

Keywords: *E. coli*; Microbiota; HS-GC/MS; Pinene; Propolis

2. INTRODUCTION

Propolis is a resinous product made by *Apis mellifera* L. bees, which is used to protect the hive against insects and microorganisms (BANKOVA; POPOVA; TRUSHEVA, 2014; FERREIRA et al., 2017; GALEOTTI et al., 2018; KAMATOU et al., 2019). It is mainly constituted by resin (60%), but it also contains waxes, essential oils, vitamins and microelements (BANKOVA; POPOVA; TRUSHEVA, 2014). The propolis production occurs through the gathering of resinous and balsamic substances present in branches and leaves, and pollen, which are aggregated to bee salivary secretions, originating the propolis (REIS et al., 2017). The chemical composition of propolis is known to be very complex. The most important classes of its biologically active compounds are characterized by polyphenols, including flavonoids, phenolic acids and their esters (PELLATI; PRENCIPE; BENVENUTI, 2013). Numerous studies have revealed the versatile biological activities of propolis, including antibacterial, antifungal, antiviral, cytotoxic, antioxidant, anti-inflammatory and immunomodulatory, among others (BANKOVA; POPOVA; TRUSHEVA, 2014). The specificity of the flora at the site of collection determines the chemical composition of propolis, including the volatile compounds (BANKOVA; POPOVA; TRUSHEVA, 2014). Volatile compounds are found in very

low percentages (generally up to 1%) in propolis samples worldwide, but their aroma and significant biological activity make them important for propolis characterization (BANKOVA; POPOVA; TRUSHEVA, 2014; FERNANDES et al., 2015). On the other hand, studies focusing in essential oils of propolis volatiles are relatively scarce, most of them dealing with antimicrobial properties against fungus, Gram-positive and Gram-negative bacteria (SENA-LOPES et al., 2018).

The most used form of propolis is the ethanolic extract (EEP), but a resinous compound constituted of several substances such as wax, resins and gums is also obtained as a residue, which represents almost 80-94% of propolis (HEIMBACH et al., 2014; HEIMBACH et al., 2016; DO AMARAL DUARTE et al., 2017; DE FRANCISCO et al., 2018). The use of these residues in animal feed may contribute to the reduction of wastes, dealing with the current environmental concern, as well as provide valuable nutrients at a low cost (SANTOS et al. 2013; REIS et al., 2017).

Antibiotics are extensively used in intensive livestock industries such as swine production as growth promoters, in prophylactic or metaphylactic treatments to prevent diseases and to treat different diseases (BARTON, 2014). Antibiotic resistance in bacteria associated with pigs not only affects pig production, but it also has an impact on human health through the transfer of resistant organisms and associated genes via the food chain and it could compromise treatment of human infections (BARTON, 2014). Currently, its impact is considerable with treatments failures associated with multidrug-resistant bacteria and it has become a global concern to public health (BALOUIRI et al., 2016). With these concerns, many researchers have focused on the investigation of plant and microbial extracts, essential oil, pure secondary metabolites, and new synthesized molecules as potential antimicrobial alternatives. Plants and other natural sources can provide a huge range of complex and structurally diverse compounds, which could also reduce the bacterial resistance in cooperation with standard antibiotics due to their safety and efficiency (BALOUIRI et al. 2016; WANG et al., 2018).

Herbs, spices and their essential oils (EOs) can exert antimicrobial, coccidiostatic or anthelmintic activities in monogastric animals. EOs also exhibit antioxidant, anti-inflammatory, anti-carcinogenic, and hypolipidemic activities and could favorably affect gut functions by stimulating endogenous digestive secretions (e.g. enzymes, bile, and mucus), and maintaining intestinal epithelial structures. Thus, EOs can be used as growth promoters

in animal production (SAMANTA et al., 2017). It also demonstrates the importance of modulating the gut microbiota of young animals in order to have a healthy microbiota developed that improve the animal performance. Several *in vivo* studies have indicated that EOs increased the *Lactobacillus* group and decreased *E. coli* or total coliforms in piglets. In several poultry studies, diets supplemented with EOs led to some fundamental changes on gut microbiota mainly in the number of observed *Lactobacillus* species (OMONIJO et al., 2018).

Oxidative stress represents an important chemical mechanism that leads to biological damage, which in turn can affect growth performance and health in pigs, especially in modern high-performance swine production systems. The oxidative stress might be associated with a drop in performance, compromised immunity, muscle degeneration, increased risk of stroke in fast-growing pigs, mulberry heart disease, reduced appetite, diarrhea, destruction of liver tissue, and increased risk of abortion of gestation sows (OMONIJO et al., 2018). Synthetic antioxidants are commonly used as effective feed additives in pig diets in order to increase the stability of feed and protect nutrients (e.g. fat and vitamins) from oxidation. However, synthetic antioxidants have also become controversial due to their potential adverse effects on health (REIS et al., 2017). This has driven to the search of natural compounds that could not only replace synthetic antioxidants in pig feed, but also provide additional zootechnical benefits. It is well known that EOs and plant extracts have anti-oxidative effects and they have been used successfully in animal diets (OMONIJO et al., 2018).

In this context, this study aimed to investigate the presence of bioactive compounds in propolis residues. With this aim, the volatile composition and phenolic content of essential oils of propolis (EOP), moist residue (EOMR) and dry residue (EODR) were determined. In addition, the *in vitro* antioxidant capacity (ABTS, DPPH and FRAP) and antibacterial activity against a pathogenic (*E. coli* ATCC 25922) and a beneficial (*L. plantarum* ATCC 8014) bacterium model of these EOs were compared with that of EEP.

3. MATERIALS AND METHODS

3.1. Propolis and propolis residues

Crude organic propolis and industrial residues from ethanolic extraction of propolis were provided by Breyer – Naturais e Orgânicos (União da Vitória, Paraná State, Brazil). Crude organic propolis (CP) was obtained in pieces, whereas the moist propolis residue (MR) was obtained in a solid form soaked in ethanol and the dry propolis residue (DR) in a dried form (Figure 1.1). The moist propolis residue was dried in an oven at 60°C to eliminate all the solvent.

Figure 1.1. Flow chart of the sequences to obtain propolis residues.

3.2. Ethanolic extract of propolis (EEP)

Crude organic propolis was ground into a fine powder. Two grams were mixed with 25 mL of 80% (v/v) ethanol solution and shaken for 30 min at 70°C. After extraction, the mixture was kept overnight at -18°C, centrifuged, and filtered using qualitative filter paper 14 µm pore size to produce the ethanolic extract of propolis (EEP). EEP was freeze-dried before analysis.

3.3. Essential oil extraction

Essential oils of propolis (EOP), moist residue (EOMR) and dry residue (EODR) were obtained by hydrodistillation for 4 h in a Clevenger-type apparatus. Distillation was done using samples 5-fold diluted in distilled water. The EOs obtained were stored in amber flasks at 4°C until analysis. EOs volumes were measured to calculate the yield of the process.

3.4. Chemical composition of essential oils

The chemical composition of EOs were determined by headspace gas chromatography/mass spectrometry (HS-GC/MS) using a gas chromatograph Shimadzu 2010 coupled to a mass spectral detector Shimadzu QP 2010 Plus. EOs samples were placed in vials and heated at 40°C, with agitation for 5 min, in a heating module to release the volatile constituents. After heating, 500 µL of the gaseous phase was collected using a 2.5 mL syringe and injected with a 1:50 split ratio. The separation of volatile compounds was performed in a DB5 capillary column (30 m x 0.25 mm x 0.25 µm, J&W Scientific, Palo Alto, CA). Mass spectra and total ion currents (TIC chromatograms) were obtained by automatic scanning with energy ionization 70 eV, in the mass range m/z 35-500. The temperature ramp began at 40°C maintained for 4 min, then 150°C at 3°C per min and 250°C at 15°C per min maintained for 2 min. Helium was used as carrier gas at a linear velocity of 36.1 cm/s. The retention index (RI) was calculated for all the volatile compounds using a homologous series of C8–C20 n-alkanes (04070 Sigma-Aldrich), according to the linear equation of Van den Dool and Kratz.

Calculation of the percentages of oil components was based on measurements of normalized GC peak areas in relation to the total area of all sample constituents. Individual constituents were identified by comparison of their RI and mass spectra with data published in the literature and MS library (Wiley8 and FFNSC).

3.5. Antibacterial activity

3.5.1. Bacterial strains and growth conditions

The following model strains from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used in the antibacterial evaluations: *Escherichia coli* (ATCC 25922) as a model of pathogenic bacterium and *Lactobacillus plantarum* (ATCC 8014) as a model of beneficial bacterium.

The Minimum Inhibitory Concentration (MIC) of EOs and EEP was determined by following a broth microdilution method based on the Clinical and Laboratory Standards Institute guidelines M07-A9 (CLSI, 2012). The tests were performed in microplates containing Mueller-Hinton broth (*E. coli*) and MRS broth (*L. plantarum*).

Fifty microliters of EOs was dissolved in 25 μL of Tween 80, as an emulsifier, and broth was added to obtain 1500 μL of stock solution. The solution was poured into 96-well microplate and diluted to yield concentrations from 14.8 to 0.12 mg/mL. The dry weight of EEP equivalent to EO volume was also used to perform MIC.

The strains were suspended in saline solution (0.85% v/v) to obtain a density of 10^8 CFU/mL (0.5 McFarland turbidity standard). Subsequently, the inoculum was diluted at 1:100 to obtain a final concentration of 10^6 CFU/mL. The microplate was incubated in a microplate reader-incubator (Vitor™ X3, PerkinElmer) at 35°C for 24 h (*E. coli*) and 30°C for 36 h (*L. plantarum*). The control test was performed simultaneously, using only 25 μL of Tween 80 in the stock solution. The existence or not of bacterial growth was evaluated by construction of survival curves and by resazurin test at the end of the incubation period. The lowest concentration that did not produce detectable optical density values at 600 nm until the end of incubation was considered as the MIC obtained by survival curves. For resazurin test, 25 μL of resazurin at 0.0135% m/v were used per well. Thus, the presence of viable cells was evidenced through a change in the resazurin color from blue resazurin to pink resofurin. All assays were carried out in triplicate in three independent replicates.

3.5.2. Bacterial growth modeling and calculation of kinetics parameters

Bacterial growth kinetics (or survival curves) for EOs and EEP were built with the optical density values at 600 nm of the microplate wells measured each hour during 24 h for *E. coli* and 36 h for *L. plantarum*. Data were adjusted to the Gompertz model modified by ZWIETERING et al. (1990), with a confidence level of 95% using the Levenberg–Marquardt algorithm of STATISTICA 12.0 (StatSoft, Inc., Tulsa, OK, USA) software:

$$y = A \exp \left\{ - \exp \left[\frac{\mu_{\max} \cdot e}{D} (\lambda - t) + 1 \right] \right\}$$

Where y represents the relative population size against time; D is the asymptote (i.e the maximal bacterial culture density at 600nm); $\mu_{\max}$ represents the maximum specific growth rate (h^{-1}) and it is the tangent of the log phase curve; and λ is the lag phase duration (h) and it is defined as the x-axis intercept of this tangent. Finally, the goodness of fit for the model was determined based on the mean square error (MSE) and on the corrected determination coefficient (corrected R^2) for each set of data.

3.5.3. CFU counting

Colony-forming unit (CFU) counting was performed after the incubation period from the wells corresponding to inoculum control, Tween 80 control, and concentration of 14.6 mg/mL of EOs and EEP. An aliquot of 50 μ L was taken from ten-fold serial dilutions from these wells and culture in MH or MRS agar. Plates were incubated for 18-20 h at 35°C (*E. coli*) and 48 h at 30°C (*L. plantarum*).

3.6. Antioxidant activity

All samples of EOs and EEP were diluted in ethanol PA and homogenized by ultrasound for 45 min in a water bath at 45°C, because the difficulty to homogenize EOMR and EODR with ethanol PA.

3.6.1. ABTS free radical scavenging assay

The antioxidant capacity was determined by free radical ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) according to Al-Duais et al. (2009) with modifications. The radical ABTS stock solution was diluted with 75 mM potassium phosphate buffer (pH 7.4) and stored at room temperature for 16 h. The radical ABTS was then diluted with ethanol PA to obtain an optical density of 0.700 ± 0.020 at 734 nm. Aliquots of 30 μ L of the EOs and EEP diluted in ethanol PA were added to 3 mL of ABTS radical solution and kept in dark at room temperature. The optical density was measured after 6 min of the beginning of oxidation. Ethanol PA was used as blank and Trolox was employed as standard at concentrations ranging from 1000 to 62.5 μ M. Optical density was measured at 734 nm. The results were expressed as μ mol of Trolox equivalents (TE) per mg of sample (μ mol TE/mg).

3.6.2. DPPH free radical scavenging assay

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free scavenging activity was determined following the method described by Moraes-de-Souza et al. (2008). The reaction mixture consisted of 500 μ L of the diluted solutions of EOs and EEP, 3 mL of ethanol, and 300 μ L of

150 μM DPPH radical solution in ethanol PA. After 45 min in the dark, the optical density was measured at 517 nm. Ethanol was used as blank and a calibration curve was built with Trolox as standard at concentrations ranging from 10 to 100 μM . The results were expressed as μmol of Trolox equivalents (TE) per mg of sample ($\mu\text{mol TE/mg}$).

3.6.3. Ferric Reducing Antioxidant Power

The analysis consists in the reduction of Fe^{3+} with 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ) in an acid reaction condition. The Fe^{3+} reduced to Fe^{2+} in a complex with TPTZ increases the optical density at 595 nm. The FRAP reagent was prepared with 50 mL of buffer acetate (300 mM, pH 3.6), 5 mL of TPTZ solution (10 mM TPTZ in 40 mM HCl) and 5 mL of FeCl_3 (20 mM) in an aqueous solution. Aliquots of 120 μL of EOs and EEP were added with 180 μL of distilled water and 1.2 mL of FRAP reagent. Optical density was measured at 595 nm after 8 min of incubation at 37° C. Distilled water was used as blank. A calibration curve was plotted using ferrous sulphate as standard. The results were expressed as μM of Fe^{2+} equivalents per mg of sample ($\mu\text{M Fe}^{2+}/\text{mg}$).

3.7. Total phenolic content

Total polyphenol content of EOs and EEP was determined using the Folin-Ciocalteu spectrophotometer method described by Singleton, Orthofer and Lamuela (1999). EOs and EEP were diluted in ethanol PA and subjected to the same homogenization procedures as described in section 3.6. EOs and EEP (150 μL) were mixed with 750 μL of Folin-Ciocalteu reagent (1:10) and 600 μL of 7.5% Na_2CO_3 . Optical density was measured using a spectrophotometer UV-mini 1240 (Shimadzu-Co) at 740 nm after 2 h of incubation at room temperature in the dark. Blank was conducted at similar conditions with distilled water. A calibration curve was plotted using gallic acid as standard and the results were expressed as mg of gallic acid equivalents per g of sample (mg GAE/g).

3.8. Statistical analyses

All determinations were performed in triplicate and the results were expressed as means $\pm$ standard deviation. Statistical analyses were performed using SAS[®] with PROC GLM and Tukey's test was used as a comparison test of the means. The results were considered statistically significant when $p < 0.05$.

4. RESULTS

4.1. Essential oils yield and chemical composition

The EOs yield was 1.13% for propolis (EOP), 0.12% for moist residue (EOMR) and 0.16% for dry residue (EODR) from organic propolis extraction. Fourteen compounds in EOP (97.46%), sixteen in EOMR (75.43%), and eighteen in EODR (53.95%) were identified by HS-GC/MS analysis (Table 1.1). The major components of EOP were α -pinene (66.48%), β -pinene (18.46%), camphene (2.94%), thuja-2,4(10)-diene (2.04%), and limonene (2.04%). EOMR was mainly composed by α -pinene (42.17%), β -pinene (10.29%), thuja-2,4(10)-diene (5.02%), ethyl benzoate (4.74%), and limonene (2.72%). In EODR, α -pinene (16.25%), ethyl benzoate (7.80%), β -pinene (5.15%), thuja-2,4(10)-diene (4.67%), and limonene (3.07%) were the major compounds.

Table 1.1. Chemical composition of the EOs from crude organic propolis and its residues.

Compound	RI _{calc}	RI _{lit}	OEP (%)	OEMR (%)	OEDR (%)
Hexanal	801	801	0.19	-	-
Tricyclene	922	926	1.54	-	-
α -Thujene	931	930	1.01	0.97	1.13
α -Pinene	938	939	66.48	42.17	16.25
Camphene	952	954	2.94	-	0.55
Thuja-2,4(10)-diene	958	960	2.04	5.02	4.67
Sabinene	977	975	0.45	-	0.50
β -Pinene	981	979	18.46	10.29	5.15
Myrcene	995	990	0.67	1.32	-
n-Octanal	1005	998	-	0.6	0.35
α -Terpinene	1020	1017	0.22	0.67	0.91
p -Cymene	1027	1024	0.81	2.29	2.07
Limonene	1032	1029	2.04	2.72	3.07
γ -Terpinene	1062	1059	0.26	0.71	0.99
Acetophenone	1068	1065	0.35	0.68	0.64
n-Nonanal	1105	1100	-	0.62	0.97
Ethyl benzoate	1172	1173	-	4.74	7.80
n-Decanal	1206	1201	-	0.52	1.32
α -Copaene	1377	1376	-	0.93	-
Ethyl decanoate	1395	1395	-	-	0.74
(E)-Caryophyllene	1426	1419	-	1.18	3.02
Zonarene	1529	1529	-	-	3.82
Total			97.46	75.43	53.95

*RI_{calc}: Retention index calculated; RI_{lit}: Retention index from literature.

4.2. Antibacterial activity

The concentration of Tween 80 used to emulsify the stock solution had some antibacterial activity on the pathogen *E. coli* ATCC 25922, whereas no effect was detected on *L. plantarum* ATCC 8014. Although MIC was not observed, the highest concentration (14.8 mg/mL) of EEP and EOs was able to cause disturbance on the growth of *E. coli* and *L. plantarum* (Figure 1.2). In particular, both bacteria were highly susceptible to EEP, reaching the lowest OD₆₀₀ at the end of incubation time. The adjusted data can only describe the action of the samples until the maximum log phase.

The modified Gompertz model allowed the evaluation of these disturbances in terms of maximal bacterial culture density (D), maximum specific growth rate ($\mu_{\max}$) and lag phase duration or adaptation time (λ) (Figure 1.3). In *E. coli*, D was significantly affected ($p < 0.05$) by Tween 80 and all the propolis treatments, in particular EODR that showed the lowest D at the end of incubation time. EEP, EOP and EOMR had a similar D than Tween 80. In *L. plantarum*, the antibacterial effect of Tween 80 was not observed. However, all the propolis treatments decreased significantly ($p < 0.05$) the D , particularly EEP, EOMR and EODR.

Figure 1.3. Effect of Tween 80, ethanolic extract of propolis (EEP) and essential oils from crude propolis (EOP), moist residue (EOMR) and dry residue (EODR) on the maximal bacterial culture density (D), maximum specific growth rate ($\mu_{\max}$) and lag phase duration or adaptation time (λ) of *E. coli* (A) and *L. plantarum* (B). Columns with different letters were statistically different ($p < 0.05$).

Tween 80 seemed to increase significantly ($p < 0.05$) the $\mu_{\max}$ in *E. coli*, but no effect was observed in *L. plantarum*. In *E. coli*, EOP and EOMR were able to reduce the $\mu_{\max}$ to 0.034 h^{-1} and 0.032 h^{-1} , respectively, whereas EEP induced the highest $\mu_{\max}$ (0.224 h^{-1}) and EODR showed a similar $\mu_{\max}$ than the control. In *L. plantarum*, EEP, EOMR and EODR significantly ($p < 0.05$) reduced the $\mu_{\max}$, whereas no effect was observed for EOP.

Meanwhile, Tween 80 significantly affected ($p < 0.05$) the λ of *E. coli*, but no effect was observed in *L. plantarum*. Similarly, to Tween 80, EEP, EOMR and EODR decreased significantly ($p < 0.05$) the λ of *E. coli*, but this parameter was increased by EOP. In *L. plantarum*, EEP and EOP significantly ($p < 0.05$) increased the λ , whereas EOMR showed a negative effect. No significant differences were observed between EODR and the control.

After the incubation time, plating was performed to count the number of viable cells (Figure 1.4). Tween 80 and all propolis treatments significantly ($p < 0.05$) reduced the number of viable cells in *E. coli*. In particular, EEP and EOP were the most effective, followed by the propolis residues and Tween 80. In *L. plantarum*, the number of viable cells was not affected by Tween 80. Nevertheless, all propolis treatments significantly ($p < 0.05$) reduced cell counts, particularly EOMR and EEP.

Figure 1.4. Counts of viable cells (log cfu/mL) of *E. coli* (A) and *L. plantarum* (B) after the incubation time. Columns with different letters were statistically different ($p < 0.05$).

4.3. Phenolic content and antioxidant activity

EEP had the highest content in phenolic compounds (136.99 mg GAE/g) (Figure 1.5). In contrast, the EOs of propolis and its residues contained a similar concentration in phenolic compounds, but it was approximately 3-fold less than EEP. EEP also showed the highest antioxidant capacity in terms of ABTS (3.12 $\mu\text{mol TE/mg}$) and DPPH (0.15 $\mu\text{mol TE/mg}$) free radical scavenging. Meanwhile, EOs had a low antioxidant activity by ABTS, and they were not capable to scavenge the DPPH radical. In FRAP analysis, EEP also exhibited the highest activity (1386.14 $\mu\text{mol Fe}^{2+}/\text{mg}$), followed by EOMR (462.67 $\mu\text{mol Fe}^{2+}/\text{mg}$), EODR (246.48 $\mu\text{mol Fe}^{2+}/\text{mg}$) and EOP (183.98 $\mu\text{mol Fe}^{2+}/\text{mg}$).

Figure 1.5. Phenolic content and antioxidant activity of EEP and EOs in terms of ABTS and DPPH free radical scavenging and ferric reducing antioxidant power (FRAP). Columns with different letters were statistically different ($p < 0.05$).

5. DISCUSSION

The extraction of essential oils from residues generated during the propolis ethanolic extraction could increase the sustainability of the propolis chain by adding value to these residues and reducing the environmental pollution. These essential oils from propolis residues have an interesting potential to be used in animal nutrition as a natural alternative to synthetic products currently applied. In fact, the utilization of synthetic antibiotics has raised concerns about the emerging of multidrug-resistant bacteria and its impact on human health through the transfer of resistant organisms and associated genes via the food chain that could compromise the treatment of human infections (BARTON, 2014). Therefore, the application and use of these residues in animal feed may contribute to the reduction of waste and environmental pollution, as well as providing nutrients at a low cost that reduce the production costs (SANTOS et al. 2013; REIS et al., 2017).

The essential oils extracted from organic propolis and its residues presented low yield, being in concordance with results previously reported in Brazilian propolis. Thus, SENA-LOPES et al. (2018) obtained a yield of 0.25% of essential oil of Brazilian red propolis. FERNANDES et al. (2015) reported a yield of 0.07% of propolis essential oil from the Brazilian cerrado biome.

SIMIONATTO et al. (2012) achieved a yield of 3.8% of propolis essential oil from Rio Grande do Sul State (Brazil). OLIVEIRA et al. (2010) obtained an essential oil yield of 0.06% from Rio de Janeiro State (Brazil) propolis. In Venezuela, RIOS et al. (2014) also reported a yield of 0.06% of essential oil propolis. Meanwhile, PELLATI; PRENCIPE; BENVENUTI (2013) obtained a yield of 0.13% from Italian propolis. In this study, EO from crude propolis showed a higher yield than the EOs from residues. In addition, EOMR and EODR were not limpid and highly viscous at ambient temperature.

Eos of propolis and its residues were mainly composed by α -pinene, β -pinene, and limonene, although different concentrations were detected in each EO. In addition, nine compounds were present in all propolis EOs, but the processing of propolis residues probably caused changes in the volatile composition. The EOMR was obtained after the extraction and drying of propolis at 60°C, whereas EODR was produced after the drying and withdrawal of the wax. These biochemical changes in propolis residues could be similar than those occurred during the drying of herbs, including alterations in the aroma generated by the loss of volatiles or the formation of new volatiles by oxidative reactions or esterification reactions (HOSSAIN et al., 2010).

Eos of propolis analyzed in this study showed a quite similar composition than that of crude Brazilian green propolis characterized by NUNES; GUERREIRO (2012) using HS-GC/MS and ESI-MS, being also β -pinene and α -pinene the major compounds. KAMATOU et al. (2019) identified by HS technique that propolis from various localities of South Africa was predominantly composed by α -pinene (1.2-46.5%), β -pinene (2.0-21.8%), limonene (trace-11.6%), 1,8-cineole (0.1-11.0%), and α -thujene (trace-11.0%). KAŠKONIENE et al. (2014) also detected by static headspace GC-MS high amounts of α -pinene and β -pinene in propolis from China, Uruguay, Estonia and Brazil. Meanwhile, PELLATI; PRENCIPE and BENVENUTI (2013) reported low level of monoterpenes in nine Italian propolis using headspace solid phase micro extraction, except in one sample from Southern Italy which had 13.19% of α -pinene.

As shown, the use of solvent-free headspace (HS) technique allows the extraction of low boiling point compounds or volatile constituents as those present in propolis resins (KAMATOU et al., 2019). However, many studies determined the EOP chemical composition by using only GC/MS technique. Thus, SENA-LOPES et al. (2018) identified in EO of Brazilian red propolis 13.1% methyl eugenol, 2.5% (E)- β -farnesene and 2.3% δ -amorphene.

FERNANDES et al. (2015) showed that the EO of propolis from the Brazilian cerrado biome was mainly composed by (E)-caryophyllene (7.85%), δ -cadinene (7.67%), spathulenol (6.65%), viridiflorene (4.52%), α -copaene (4.01%), aromadendrene (3.85%), α -trans-bergamotene (3.73%) and (E)-nerolidol (3.72%). OLIVEIRA et al. (2010) reported that the most abundant compounds in propolis from Rio de Janeiro State (Brazil) were β -caryophyllene (12.7%), acetophenone (12.3%) and linalool (6.47%). SIMIONATTO et al. (2012) identified the predominance of the monoterpenes α -pinene (57-63%), β -pinene (12.5-30.8%) and limonene (1.5-11.2%) in EO propolis from Rio Grande do Sul State (Brazil), the same major compounds detected in this study. High amounts of α -pinene and β -pinene were also detected in the volatile oil of propolis from different Brazilian regions (BANKOVA; POPOVA; TRUSHEVA, 2014). The volatile chemical composition of propolis can change with the geographical origin due to variations in the flora, weather pattern and the type of bee involved in the pollination process, among others (BANKOVA; POPOVA; TRUSHEVA, 2014; KAMATOU et al., 2019).

The antimicrobial activity of EEP was similar than that of the ethanolic extract of organic propolis from Brazilian south region analyzed by TIVERON et al. (2016). In fact, these authors could not determine the MIC against *Escherichia coli* ATCC 25922 at the maximum concentration evaluated (i.e. 12.5 μ g/mL). However, in the present study was possible to observe that EOs of propolis and its residues had a much stronger effect against the pathogenic *E. coli* than on the beneficial *L. plantarum*. This selective antibacterial activity enhanced the possible application of the EOs of propolis in animal production. On the other hand, EEP showed stronger antibacterial activity on both bacterium models than the EOs of propolis.

SIMIONATTO et al. (2012) concluded that the monoterpene composition contributes to the biological activity of volatile oil of propolis, inferring that α -pinene, β -pinene and limonene, are responsible for biological effects of propolis EOs. In fact, these compounds are already known to play an important inhibitory effect on different bacteria (MAGIATIS et al., 1999; BURT, 2004; MELLIOU et al., 2007). LEITE et al. (2007) observed the intense antimicrobial potential of eugenol, β -pinene and α -pinene, which were able to inhibit significantly the growth and cell viability of potential infectious endocarditis caused by Gram-positive bacteria. Other studies that identified α -pinene as a major constituent in EOs from plants also have observed antibacterial activity against Gram-negative and Gram-positive bacteria

(REBOUÇAS DE ARAÚJO et al., 2017). However, UTEGENOVA et al. (2018) studied the specific enantiomers of α -pinene and sabinene as racemic mixtures and the (-)-enantiomer of β -pinene, available from commercial sources, and observed that the individual constituents ($\pm$)- α -pinene and (-)- β -pinene showed low activity. There is some evidence that minor components also play an important role in the antibacterial activity, producing synergistic, antagonistic or additive effects on other components (BURT, 2004). In fact, it was reported that the whole EO have a greater antibacterial activity than the major components acting alone (BRENES; ROURA, 2010). Considering the large number of different groups of chemical compounds present in EOs, it is mostly likely that their mode of action includes several targets in the bacterial cell (BURT, 2004). An important characteristic of EOs and their components is their hydrophobicity, which enables them to partition lipids in the bacterial cell wall and mitochondria, disturbing the structures and rendering them more permeable (BRENES; ROURA, 2010).

Some EOs have shown a selective influence on the gut microflora. A mixture of carvacrol, cinnamaldehyde and capsicum oleoresin increased the population of *Lactobacilli* and the ratio of *Lactobacilli* to *Enterobacteria* in the jejunum and cecum of early-weaned piglets (ZHAI et al., 2018). The *in vitro* study also showed that cinnamaldehyde was highly inhibitory against coliform bacteria and *E. coli*, whereas it hardly inhibited the growth of *Lactobacilli*. Si et al. (2006) demonstrated *in vitro* that EOs exhibited a high efficacy against the pathogens *Salmonella typhimurium* DT 104, *E. coli* O157:H7 and *E. coli* K88, but a low inhibition of the beneficial bacteria *Lactobacillus* and *Bifidobacterium* in mediums containing pig cecal digesta. Ouwehand et al. (2010) concluded that the combination of thymol and cinnamaldehyde had the best potential to control the proliferation of pathogenic bacteria and maintain a proper gut health. Li et al. (2012) also observed that the addition of thymol and cinnamaldehyde improves the feed intake, growth rate and feed conversion ratio of weaner piglets, whereas reduces the incidence of diarrhea. These EO compounds also modulated the microbial populations present in the feces, with a reduction of *E. coli* and an increase of *Lactobacillus* counts. This study has shown that thymol and cinnamaldehyde were effective in improving the animal immune status, which is important during the weaning period when piglets are extremely sensitive to disease infection.

Our results for EEP were within the range of antioxidant activity that have been observed by Tiveron et al. (2016). In contrast, the EOs of propolis and its residues presented weaker

antioxidant activity than EEP in terms of ABTS, DPPH and FRAP. These findings are comprehensive considering that the EO of propolis is just a fraction of the complex composition of the propolis. In addition, data evidenced that ethanolic extraction is a process that successfully remove the waxy material of propolis resin and preserve the fraction of polyphenols, which are considered to contribute to antioxidant properties (KARLA et al., 2017). Many studies make a positive correlation between the phenolic content and antioxidant activity, being in concordance with study (BRENES; ROURA, 2010). Meanwhile, the lower phenolic content of the EOs of propolis and its residues resulted in lower antioxidant activity. In fact, only FRAP analysis allowed to detect some antioxidant activity in the EOs, as they were able to reduce Fe^{+3} in Fe^{+2} . This low phenolic content can be explained by the modest volatility and partial water-solubility of phenolic components that were partly lost during hydrodistillation (AMORATI; FOTI; VALGIMIGLI, 2013).

Phenolic compounds meet the structural requirements of free radical scavengers, and hence, they can be used as food antioxidants (OLSZOWY; DAWIDOWICZ, 2016). Antioxidant properties in some of EOs are due to the inherent ability of phenolic compounds to stop or delay the aerobic oxidation of organic matter. However, the procedure to obtain EOs from raw material limits the content of phenolics in the final matrix because many compounds are non-volatile (AMORATI; FOTI; VALGIMIGLI, 2013). In this study, EOs of propolis and its residues presented a rich composition in terpenes (α -pinene, β -pinene and limonene), which are involved in the antioxidant activity of EOs, but this characteristic was not exhibited in our samples. Similar results were obtained with rosemary EO, which was mainly composed by α -pinene and limonene, but it showed a low antioxidant activity (BARATTA et al., 1998). Alpha-pinene, beta-pinene and limonene are highly oxidizable compounds, which limit their antioxidant properties (AMORATI; FOTI; VALGIMIGLI, 2013). Lee et al. (2002) concluded that rosemary EO did not have the antioxidant effect of rosemary hydroalcoholic extracts due to the non-volatile phenols were completely removed during EO preparation. The same conclusion can be inferred to the EEP and EOs of crude propolis and the residues of propolis from the ethanolic extraction.

Essential oils have been employed in animal diets for their antimicrobial, antibacterial, antioxidant and digestive stimulant properties, being a suitable alternative to antibiotics to improve the performance and health of animals (DING et al., 2017). Based on the literature, EOs may improve animal health through several mechanisms such as direct suppression of

the proliferation of pathogens, alteration of gut microbial populations, and enhancement of immune functions (SAMANTA et al., 2017; LIU et al., 2018; KONÉ et al., 2019).

6. CONCLUSIONS

The EOs extracted from organic propolis and its residues presented low yield. These EOs were mainly composed by α -pinene, β -pinene, and limonene. The EOs were not able to scavenge stable ABTS and DPPH radicals, but they were capable to chelate transition metals as observed in FRAP assay. Nevertheless, the EOs of propolis and its residues presented a selective action against the pathogenic bacteria *E. coli*, which could benefit the gut microflora during an antibiotic treatment in swine production.

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Chapter 2. Antioxidant and antibacterial activity of phenolic acids from residues generated during the ethanolic extraction of organic propolis

1. ABSTRACT

After the ethanolic extraction of propolis, a great amount of an undissolved resinous by-product is also generated, which is mainly composed by wax, resins and gums, but it also could contain some bioactive compounds. This study aimed to evaluate the antioxidant and antimicrobial properties of free and bound phenolic compounds produced by other solvent extractions and alkaline/acid hydrolysis of these propolis residues to be applied in animal feed. With this aim, total phenolic content, antioxidant capacity in terms of ABTS, DPPH and FRAP, and antibacterial activity against a pathogenic (*Escherichia coli*) and a beneficial (*Lactobacillus plantarum*) of the phenolic acids fractions obtained were determined. The tetrahydrofuran extraction was efficient to release free phenolic acids (FPA) from crude organic propolis and its residues, but a low efficiency was observed with bound phenolic acids. The FPA fraction of dry residue (DR) showed higher phenolic content (95.77 mg GAE/g) than that of crude propolis (CP) (15.59 mg GAE/g) and moisture residue (MR) (13.60 mg GAE/g). FPA fraction of DR also exhibited higher antioxidant activity than that of CR and MR in terms of ABTS (1.21 $\mu\text{mol TE/mg}$), DPPH (0.12 $\mu\text{mol TE/mg}$) and FRAP (878.51 $\mu\text{mol Fe}^{2+}/\text{mg}$). Meanwhile, FPA of MR showed lower MIC against *E. coli* (7.4 mg/mL) than that of CP and DR (14.8 mg/mL each). Nevertheless, the concentrations of FPA tested were not able to inhibit the growth of *L. plantarum*. In addition, FPA fractions caused higher disturbance on the growth of *E. coli* than *L. plantarum*. Data obtained in this study demonstrated that propolis residues contain some bioactive compounds that could be added in animal feed to modify the gut microbiota favorably.

Keywords: *E. coli*; Microbiota; *Lactobacillus*; Propolis residue; Phenolic acids

2. Introduction

Bees have the ability to collect plant compounds that can protect the beehive against microorganisms. Propolis is one of the most fascinating bee products, being used as building material and defensive substance (REIS et al., 2017; FERNANDES et al., 2015; BANKOVA; POPOVA; TRUSHEVA, 2014). The compounds present in propolis resin come from substances actively secreted by plants or exuded from plant wounds (lipophilic materials on leaves and leaf buds, resins, mucilage, gums, among others) and collected by bees, substances produced during bee metabolism, and materials added during propolis elaboration. Thus, the composition of the plant source determines the chemical composition of propolis (ORYAN; ALEMZADEH; MOSHIRI, 2018; POPOVA et al., 2017; SENA-LOPES et al., 2018; BANKOVA; POPOVA; TRUSHEVA, 2014; MIGUEL; ANTUNES, 2011; NOGUEIRA et al., 2007).

The complex form of propolis enables the utilization of different extraction solvents such as ethanol, methanol and water. However, ethanolic extract of propolis (EEP) is the most produced because it is rich in phenolic acids and flavonoids (DARENDELIOGLU et al., 2016; MIGUEL; ANTUNES, 2011). The antioxidant and anti-inflammatory activities as well as other biological functions of polyphenols have been largely attributed to the particular chemical structures. The aromatic feature and highly conjugated system with multiple hydroxyl groups make these compounds good electron or hydrogen atom donors, and capable to break radical chain reactions and chelate metals (BONOLI et al., 2004; GIADA, MANCINI-FILHO, 2006; SOARES et al., 2008; ZHANG; TSAO, 2016; DO AMARAL DUARTE et al., 2017).

The ethanolic solution obtained during propolis extraction can be used as a final dosage form or incorporated into foods, beverages, medicines or cosmetics. However, 80-94% of propolis remains undissolved in the resinous by-product, being composed by several substances such as wax, resins and gums (ROSSETO et al., 2017), which cannot be used directly in the food industry (DE FRANCISCO et al., 2018; REIS et al., 2017; HEIMBACH et al., 2014). There is an economic, social and scientific interest in the valorization of these residues, because the expansion of the propolis market (a growth rate of 3.50% until 2021) and production (from 2300 t in 2015 to 2900 t in 2021) (DE FRANCISCO et al., 2018).

Extraction of phenolic compounds is influenced by their chemical nature, extraction method, sample particle size, storage time and conditions, as well as the presence of interfering substances (NACZK; SHAHIDI, 2004). The chemical nature of phenolics vary from simple to highly polymerized, but also form complexes with carbohydrates, proteins and other components and may be quite insoluble (MONENTE et al., 2015; NACZK; SHAHIDI, 2004). Therefore, additional steps may be required for the removal of unwanted phenolic and non-phenolic substances such as waxes, fats, and terpenes (NACZK; SHAHIDI, 2004). Alkaline, acid or enzymatic hydrolysis methods can be used to release bound phenolic compounds (SU et al., 2014; KHODDAMI; WILKES; ROBERTS, 2013).

Solubility of phenolic compounds is governed by the polarity of solvent used, degree of polymerization of the phenolics, interaction between phenolics and other food constituents and formation of insoluble complexes. Methanol, ethanol, acetone, water, ethyl acetate and, to a lesser extent, propanol, dimethylformamide and their combinations are frequently used for the extraction of phenolic compounds (NACZK; SHAHIDI, 2004).

Free or simple conjugates of polyphenols are absorbed in the upper gastrointestinal tract, whereas the unabsorbed phenolics and those bound to other materials can be further metabolized or released by the gut microbiota in the colon. These metabolites or bound phenolics can influence positively the local inflammatory status, or act indirectly as prebiotics that promote the growth of probiotics, leading to an improved gut health (OBOH et al., 2017; ZHANG; TSAO, 2016; MONENTE et al., 2015).

Phenolics are recognized as direct antioxidants, but they may also exhibit indirect antioxidant effects through the induction of endogenous protective enzymes, and beneficial regulatory effects on signaling pathways (STEVENSON AND HURST, 2007). In general, phenolic compounds are poorly absorbed in the small intestine, being estimated that 90-95% of dietary phenolics are accumulated in the colon (CLIFFORD, 2004). In the gut, phenolics may selectively suppress or stimulate the growth of some components of the intestinal microbiota, thus affecting the bacterial population dynamics (CUEVA et al., 2010; TZOUNIS et al., 2008).

Inferring that the residues of ethanolic propolis extraction could still contain some interesting bioactive compounds, this material was submitted to other solvent extractions and alkaline/acid hydrolysis methods to release free and bound phenolic compounds. The total phenolic acid content, antioxidant capacity and antibacterial activity against a pathogenic and a beneficial bacterium were then determined in the phenolic acid fractions obtained.

3. MATERIALS AND METHODS

3.1. Propolis and propolis residues

Crude organic propolis and industrial residues from ethanolic propolis extraction were provided by Breyer – Naturais e Orgânicos (União da Vitória, Paraná State, Brazil). Crude organic propolis (CP) was obtained in pieces, the moist propolis residue (MR) in a solid form soaked in ethanol and the dry propolis residue (DR) in a dried form (Figure 1.1). The moist propolis residue was dried in an oven at 60°C to eliminate all the solvent.

3.2. Ethanolic extract of propolis (EEP)

Crude organic propolis was ground into a fine powder. Two grams were mixed with 25 mL of 80% (v/v) ethanol solution and shaken for 30 min at 70°C. After extraction, the mixture was kept overnight at -18°C, centrifuged, and filtered using a qualitative filter paper of 14 µm pore size to produce the ethanolic extract of propolis (EEP). EEP was freeze-dried before analysis.

3.3. Phenolic acid fractions

The procedure used for the extraction and characterization of the phenolic acid fractions obtained from crude organic propolis and the residues generated after the ethanolic extraction is detailed in Figure 2.1. The extraction was performed following the method described by Jardini et al. (2010) based in Krygier (1982). The phenolic acid fractions were characterized as free phenolic acids (FPA), soluble esterified (SEPA), and insoluble esterified (IEPA).

Figure 2.1. Flow chart of the extraction and characterization of the phenolic acid fractions.

3.3.1. Extraction of free phenolic acids (FPA)

FPA from CP, MR and DR were obtained through five consecutive extractions using 25 mL of tetrahydrofuran (THF) with 15 g of sample. Each extraction was homogenized in vortex for 5 min. The supernatants were put together and filtered with anhydrous sodium sulfate. The solvent was evaporated in a vacuum evaporator at 40°C and suspended in 25 mL of

methanol. The FPA fractions were kept in amber flasks under refrigerated conditions until analysis.

3.3.2. Extraction of soluble esterified phenolic acids (SEPA)

The residues from FPA extraction were subjected to six consecutive extractions with 20 mL of a mixture of methanol/acetone/water (7:7:6). Each extraction was homogenized in vortex for 5 min. The extracts were then centrifuged for 10 min at 10,000 rpm, and the supernatants were used to obtain the SEPA of each sample. Solvents were evaporated at 40°C, the volume of the remaining aqueous fraction was measured and the same volume of 4 N sodium hydroxide was added. After 4 h of alkaline hydrolysis at room temperature in the dark with constant agitation, the pH was corrected to 2 with 6 N hydrochloric acid and samples were centrifuged for 10 min at 10,000 rpm. The supernatant was then transferred to a separation funnel and subjected to six manual agitations with 15 mL of hexane to eliminate the free fatty acids and other contaminants. The phenolic acids from the aqueous fraction were extracted through six consecutive manual agitations with 15 mL of a mixture of ethyl ether/ethyl acetate/tetrahydrofuran (EE/EA/THF) (1:1:1) in separation funnels. The under fraction was filtered with anhydrous sodium sulfate. The solvent was evaporated at 40°C and suspended in 25 mL of methanol. The SEPA fractions were kept in amber flasks under refrigerated conditions until analysis.

3.3.3. Extraction of insoluble esterified phenolic acids (IEPA)

IEPA were obtained from the residues generated during the SEPA extraction. These residues were firstly hydrolyzed with 25 mL of 4N sodium hydroxide and then subjected to the same steps described in section 3.3.2 to obtain SEPA.

3.4. Total phenolic content

Total polyphenol content of each phenolic acid fraction was determined using the Folin-Ciocalteu spectrophotometer method described by Singleton, Orthofer and Lamuela (1999). Volumes of 150 μ L of phenolic acid fractions previously diluted in ethanol PA were mixed with 750 μ L of 10% Folin-Ciocalteu reagent and 600 μ L of 7.5% Na_2CO_3 . Optical density was

measured using a spectrophotometer UV-mini 1240 (Shimadzu-Co) at 740 nm after 2 h of incubation at room temperature in the dark. Blank was conducted in the same conditions with distilled water. A calibration curve was plotted using gallic acid as standard and the results were expressed as mg of gallic acid equivalents per g of sample (mg GAE/g).

3.5. Antioxidant activity

3.5.1. ABTS free radical scavenging assay

The antioxidant capacity was determined in terms of ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) free radical scavenging by the method proposed by Al-Duais et al. (2009) with some modifications. The stock solution of radical ABTS was diluted with 75 mM potassium phosphate buffer (pH 7.4) and stored at room temperature for 16 h. Then, the ABTS radical solution was adjusted in ethanol PA to an optical density of 0.700 ± 0.020 at 734 nm. Aliquots of 30 μ L of phenolic acid fractions previously diluted in ethanol PA were added with 3 mL of ABTS radical solution and kept in the dark at room temperature. The optical density was measured after 6 min at 734 nm. Ethanol PA was used as blank and Trolox was employed as standard at concentrations ranging from 1000 to 62.5 μ M. Results were expressed as μ mol of Trolox equivalents (TE) per mg of sample (μ mol TE/mg).

3.5.2. DPPH free radical scavenging assay

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free scavenging activity was assessed following the method described by Moraes-de-Souza et al. (2008). The reaction mixture consisted of 500 μ L of diluted solutions of phenolic acid fractions, 3 mL of ethanol PA, and 300 μ L of 150 μ M DPPH radical solution in ethanol PA. After 45 min in the dark, the optical density was measured at 517 nm. Ethanol PA was used as blank and a calibration curve was built with Trolox at concentrations ranging from 10 to 100 μ M. Results were expressed as μ mol of Trolox equivalents (TE) per mg of sample (μ mol TE/mg).

3.5.3. Ferric Reducing Antioxidant Power

This assay consists in the reduction of Fe^{3+} with 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ) to a Fe^{2+} form under acidic conditions, increasing the optical density of this blue coloring complex at 595 nm. The FRAP reagent was prepared with 50 mL of buffer acetate (300 mM, pH 3.6), 5 mL of TPTZ solution (10 mM TPTZ in 40 mM HCl) and 5 mL of FeCl_3 (20 mM) in aqueous solution. Aliquots of 120 μL of phenolic acid fractions were added with 180 μL of distilled water and 1.2 mL of FRAP reagent. Optical density at 595 nm was measured after 8 min of incubation at 37°C. Distilled water was used as blank and a calibration curve was plotted using ferrous sulphate as standard ranging from 100 to 700 μM . Results were expressed as μM of Fe^{2+} equivalents per mg of sample ($\mu\text{M Fe}^{2+}/\text{mg}$).

3.6. Antibacterial activity

All phenolic acid fractions were previously dried in a desiccator to eliminate the solvent/water content. A stock solution of each phenolic acids fraction was then prepared adding 44 g of sample to 25 μL of the emulsifier Tween 80 and completed until 1.5 mL with the correspondent bacterial broth.

3.6.1. Bacterial strains

Escherichia coli ATCC 25922 was used as a model of pathogenic bacterium, whereas *Lactobacillus plantarum* ATCC 8014 was used as a model of beneficial bacterium. Both strains were provided by the American Type Culture Collection (ATCC, Rockville, MD, USA).

3.6.2. Minimal Inhibitory Concentration (MIC)

A broth microdilution method based on the Clinical and Laboratory Standards Institute guidelines M07-A9 (CLSI, 2012) was employed to determine the Minimum Inhibitory Concentration (MIC) of phenolic acid fractions.

The strains were adjusted to a 0.5 McFarland turbidity in 0.85% (v/v) saline solution to obtain a density of 10^8 CFU/mL approximately. Subsequently, the inoculum was 100-fold diluted in Mueller-Hinton broth for *E. coli* or MRS broth for *L. plantarum* to obtain a final

concentration of 10^6 CFU/mL approximately. Volumes of 20 μ L of the inoculum was exposed to each phenolic acids fraction and EEP at concentrations ranging from 0.12 to 14.8 mg/mL in 96-well microplates. Microplates were then incubated in a microplate reader-incubator (Vitor™ X3, PerkinElmer) at 35°C for 24 h (*E. coli*) and 30°C for 36 h (*L. plantarum*), measuring each hour the optical density at 600 nm. A control with 25 μ L of Tween 80 diluted in broth was also included. MIC was established as the lowest concentration of sample that inhibited the bacterial growth. All assays were carried out in triplicate in three independent experiments.

3.6.3. Bacterial growth modeling and calculation of kinetics parameters

Bacterial growth kinetics (or survival curves) for phenolic acid fractions was built using the optical density readings at 600 nm carried out every hour during 24 h for *E. coli* and 36 h for *L. plantarum*. Data were fitted to the Gompertz model modified by ZWIETERING et al. (1990), with a confidence level of 95% using the Levenberg–Marquardt algorithm of STATISTICA 12.0 (StatSoft, Inc., Tulsa, OK, USA) software:

$$y = A \exp \left\{ - \exp \left[\frac{\mu_{\max} \cdot e}{D} (\lambda - t) + 1 \right] \right\}$$

Where y represents the relative population size against time; D is the asymptote (i.e the maximal bacterial culture density at 600nm); $\mu_{\max}$ represents the maximum specific growth rate (h^{-1}) and it is the tangent of the log phase curve; and λ is the lag phase duration (h) and it is defined as the x-axis intercept of this tangent. Finally, the goodness of fit for the model was determined based on the mean square error (MSE) and on the corrected determination coefficient (corrected R^2) for each set of data.

3.7. Statistical analyses

All determinations were performed in triplicate and the results were expressed as means $\pm$ Standard Deviation. Statistical analyses were performed using SAS® with PROC GLM and Tukey's test was used as a comparison test of the means. The results were considered statistically significant when $p < 0.05$.

4. RESULTS

4.1. Free and bound phenolic acid extraction

The tetrahydrofuran extraction was able to extract the FPAs of crude organic propolis and its residues. However, the extraction of bound phenolic acids (i.e SEPA and IEPA) were not effective. The FPA fraction of DR showed a significantly ($p < 0.05$) higher phenolic content (95.77 mg GAE/g) than CP (15.59 mg GAE/g) and MR (13.60 mg GAE/g), but lower than EEP (145.35 mg GAE/g) (Figure 2.2).

Figure 2.2. Phenolic content of the ethanolic extract of propolis (EEP) and free and bound phenolic acids of crude propolis and its moist and dry residues. FPA: Free phenolic acid; SEPA: Soluble esterified phenolic acid; IEPA: Insoluble esterified phenolic acid. *not significant value.

4.2. Antioxidant activity

As shown in Figure 2.3, only FPA fractions of the samples showed antioxidant activity in terms of ABTS and DPPH free radical scavenging and ferric reducing antioxidant power (FRAP). FPA of DR exhibited a significantly ($p < 0.05$) higher antioxidant activity in terms of ABTS (1.21 $\mu\text{mol TE/mg}$), DPPH (0.12 $\mu\text{mol TE/mg}$) and FRAP (878.51 $\mu\text{mol Fe}^{2+}/\text{mg}$) than CR and MR. However, EEP exhibited the highest antioxidant activity in terms of ABTS and DPPH (3.28 and 0.15 $\mu\text{mol TE/mg}$, respectively) and the highest iron chelating power (1527.59 $\mu\text{mol Fe}^{2+}/\text{mg}$).

Figure 2.3. ABTS and DPPH free scavenging and ferric reducing antioxidant power (FRAP) of the ethanolic extract of propolis (EEP) and free and bound phenolic acids of crude propolis and its moist and dry residues. FPA: Free phenolic acid; SEPA: Soluble esterified phenolic acid; IEPA: Insoluble esterified phenolic acid. *not significant value.

4.3. Antibacterial activity

MIC was determined on *E. coli* at concentrations of 14.8 mg/mL of FPA of CP and DR and 7.4 mg/mL of MR, but none concentration of the FPA fractions were able to inhibit the growth of *L. plantarum*. Nevertheless, all FPA fractions of the samples were able to cause

disturbances in the growth of both bacteria, although higher alterations were observed in *E. coli* than *L. plantarum* (Figure 2.4 and 2.5). Therefore, only the growth kinetics parameters of each FPA fraction were calculated to observe the action trends and to find a mathematical function that could describe these behaviors. The adjusted data could only describe the action of the samples until the maximum log phase. The effect of EEP on growth kinetics parameters was not analyzed because it was already studied in chapter 1 and it was not the main objective of this study. The modified Gompertz model allowed describing the disturbances on the bacterial growth through the assessing of the maximal bacterial culture density (D), maximum specific growth rate ($\mu_{\max}$) and lag phase duration or adaptation time (λ) at each FPA concentration. Table 2.1 and 2.2 showed the mathematical function that describes the effect of FPAs as well as their level of adjustment. In *E. coli*, the use of Tween 80 caused a significant disturbance on all growth parameters, but a different effect was observed in *L. plantarum*. All concentrations of FPAs of CP, MR and DR reduced significantly ($p < 0.05$) the parameter D in both bacteria (Figure 2.6 and 2.7). The bacterial culture density decreased with the increasing of the FPA concentrations. FPA concentrations of 14.8 and 7.4 mg/mL of MR affected negatively ($p < 0.05$) the $\mu_{\max}$ of *E. coli* (Figure 2.6). In contrast, no effect was observed with the FPAs of DR, whereas FPAs of CP increased significantly ($p < 0.05$) the $\mu_{\max}$ of *E. coli*, particularly at concentrations of 3.7 mg/mL. In *L. plantarum*, all FPA concentrations of CP and MR decreased ($p < 0.05$) its $\mu_{\max}$, whereas FPAs of DR did not affect this growth parameter (Figure 2.9). Thus, the increasing of the FPA concentrations of CP and MR reduced the bacterial culture density. In *E. coli*, all FPA concentrations of CP, MR and DR affected negatively ($p < 0.05$) the λ , a similar effect was observed with the increase of FPA concentrations (Figure 2.6). In contrast, all FPA concentrations of MR and most of DR (3.7-14.8 mg/mL) increased significantly ($p < 0.05$) the λ of *L. plantarum* (Figure 2.7). Nevertheless, a similar effect was observed between the different FPA concentrations of MR, whereas the λ of *L. plantarum* increased with the increment of the FPA concentrations of DR. Meanwhile, none of the FPA concentrations of CP showed a significant effect on the λ of *L. plantarum*.

Figure 2.4. Effect of FPA fractions of crude propolis (A), moist residue (B) and dry residue (C) on the growth of *E. coli*. Dotted curves are optical density data with standard deviations and dashed curves are the adjusted data using the modified Gompertz model. CP: Crude propolis; MR: Moist residue; DR: Dry residue.

Figure 2.5. Effect of FPA fractions of crude propolis (A), moist residue (B) and dry residue (C) on the growth of *L. plantarum*. Dotted curves are optical density data with standard deviations and dashed curves are the adjusted data using the modified Gompertz model. CP: Crude propolis; MR: Moist residue; DR: Dry residue.

Table 2.1. Parameter value with standard deviation (PV) of the maximal bacterial culture density (D), maximum specific growth rate ($\mu_{\max}$) and lag phase (λ) of *E. coli* at FPA concentrations of 14.8 mg/mL of crude propolis, moist residue and dry residue, and equations (EQ) that describe their effect on the growth parameters indicating the level of adjustment (ADJ).

FPA		D	$\mu_{\max}$ (h ⁻¹)	λ (h)
Crude propolis	PV	0.059 ± 0.006	0.113 ± 0.010	3.63 ± 0.063
	EQ	D=0.86*exp(-0.27*conc)	$\mu_{\max} = ((0.53) + ((-0.33) * \text{conc}^{0.1}) + (-0.46 * \exp(\text{conc})))$	$\lambda = ((3.85 * \exp(-1.40 * \text{conc})) + (3.21 * \exp(0.008 * \text{conc})))$
	ADJ	R ² = 0.955	R ² = 0.922	R ² = 0.959
Moist residue	PV	0.049 ± 0.006	0.014 ± 0.002	2.72 ± 0.144
	EQ	D=0.88*exp(-0.45*conc)	$\mu_{\max} = (0.07 / (1 + \exp(-1.15))) * (7.20 - \text{conc})$	$\lambda = ((2.53 * \exp(-11.76 * \text{conc})) + (4.52 * \exp(-0.030 * \text{conc})))$
	ADJ	R ² = 0.984	R ² = 0.883	R ² = 0.949
Dry residue	PV	0.148 ± 0.005	0.063 ± 0.007	3.21 ± 0.031
	EQ	D=0.89*exp(-0.59*conc)	No effect	$\lambda = ((3.36 * \exp(-1.75 * \text{conc})) + (3.70 * \exp(-0.009 * \text{conc})))$
	ADJ	R ² = 0.952	-	R ² = 0.952

Table 2.2. Parameter value with standard deviation (PV) of the maximal bacterial culture density (D), maximum specific growth rate ($\mu_{\max}$) and lag phase (λ) of *L. plantarum* at FPA concentrations of 14.8 mg/mL of crude propolis, moist residue and dry residue, and equations (EQ) that describe their effect on the growth parameters indicating the level of adjustment (ADJ).

FPA		D	$\mu_{\max}$ (h^{-1})	λ (h)
Crude propolis	PV	0.232 ± 0.040	0.016 ± 0.002	14.14 ± 0.35
	EQ	$D=1.13*\exp(-0.11*\text{conc})$	$\mu_{\max}=(0.14*\exp(-0.21*\text{conc}))$	No effect
	ADJ	$R^2 = 0.977$	$R^2 = 0.943$	-
Moist residue	PV	0.409 ± 0.018	0.035 ± 0.002	15.25 ± 0.406
	EQ	$D=1.20*\exp(-0.07*\text{conc})$	$\mu_{\max}=(0.14*\exp(-0.10*\text{conc}))$	$\lambda=(13.35+\text{conc}/0.012+(0.53*\text{conc}))$
	ADJ	$R^2 = 0.996$	$R^2=0.977$	$R^2 = 0.987$
Dry residue	PV	0.746 ± 0.014	0.144 ± 0.009	16.79 ± 0.080
	EQ	$D=1.16*\exp(-0.029*\text{conc})$	No effect	$\lambda=(13.35+\text{conc}/0.99+(0.20*\text{conc}))$
	ADJ	$R^2 = 0.972$	-	$R^2 = 0.932$

Figure 2.6. Effect of FPA of crude propolis (A), moist residue (B) and dry residue (C) at concentrations of 14.8, 7.4, 3.7 and 1.85 mg/mL on the maximal bacterial culture density (D), maximum specific growth rate (μ_{max}) and lag phase (λ) of *E. coli*.

Figure 2.7. Effect of FPA of crude propolis (A), moist residue (B) and dry residue (C) at concentrations of 14.8, 7.4, 3.7 and 1.85 mg/mL on the maximal bacterial culture density (D), maximum specific growth rate (μ_{max}) and lag phase (λ) of *L. plantarum*.

5. DISCUSSION

The search for alternative ways to use waste material is necessary, once environmental and economic issues are involved. In the case of propolis, great amounts of a resinous waste are generated during the ethanolic extraction, which has not a concrete role in other segments of the propolis chain. This waste cannot be reused for human consumption, but it could be acceptable for animal feed as some compounds of interest probably remain in these residues.

A considerable portion of propolis components responsible for biological activities is derived from plant sources. Thus, we applied in propolis residues the same extractions used for plant material to release free and bound phenolic acids. For example, Krygier et al. (1982) extracted free and esterified phenolic acids from oilseeds using a mixture of methanol-

acetone-water (7:7:6) and alkaline and acid treatment. Meanwhile, Bonoli et al. (2004) reported that alkaline and acid hydrolysis methods enabled complementary information of the bound phenolic pattern of barley. In particular, a prolonged alkaline hydrolysis seems to be a reliable method for extracting hydroxycinnamic acids, while acid hydrolysis allows higher extraction yields of generic phenols, which showed a considerable antioxidant activity. In this study, the extraction of propolis wastes with tetrahydrofuran was able to release some interesting compounds that corresponds to free phenolic acids (FPA). However, secondary extractions with unconventional solvents and alkaline and acid treatments did not successfully extract the soluble and insoluble esterified phenolic acids. The FPA extraction of dry residues (DR) enabled release higher phenolic content (95.77 mg GAE/g) than in the crude propolis (CP) and the moist residues (MR), 15.59 and 13.60 mg GAE/g respectively. The wax content of CP and MR probably reduced the yield of extraction with tetrahydrofuran in comparison with DR, which was previously submitted to a wax extraction step. FPA of DR also exhibited a higher antioxidant activity than CP and MR, in terms of ABTS and DPPH scavenging power (1.21 and 0.12 $\mu\text{mol TE/mg}$, respectively) and iron chelating power (875.51 $\mu\text{mol Fe}^{2+}/\text{mg}$), because antioxidant properties are intrinsically correlated to phenolic content (BRENES; ROURA, 2010). However, the extraction of propolis with ethanol released higher amounts of phenolic compounds (145.35 mg GAE/g) with higher antioxidant activity in terms of ABTS (3.28 $\mu\text{mol TE/mg}$) and DPPH (0.15 $\mu\text{mol TE/mg}$) free radicals scavenging and iron chelating power (1527.59 $\mu\text{mol Fe}^{2+}/\text{mg}$). In fact, it was also confirmed in this study the well-known high capacity of hydroalcoholic solution to solubilize a great percentage of polyphenols (GALEOTTI et al., 2018).

Other authors also adopted this methodology to extract free and bound phenolic acids from vegetal processing by-products and measured their antioxidant capacity by different assays. SOARES et al. (2008) studying apple juice residue observed high release of phenolic acid content (260.01 mg GAE/100g) in SEPA fraction. This fraction also exhibited the highest antioxidant activity in terms of ABTS scavenging assay (57.36 $\mu\text{mol TE/g}$). JARDINI et al. (2010) observed that pomegranate seeds also exhibit an interesting content of free and bound phenolic acids. FPA and SEPA of pomegranate seeds were more efficient to reduce the radical DPPH compared to a synthetic antioxidant (BHT).

The antibacterial activity of FPA of CP, MR and DR was evaluated on *E. coli*, a pathogenic bacterium that can unbalance the diversity and metabolic activity of the intestinal microbiota, and *L. plantarum*, a beneficial bacterium with health-promoting capacities as a probiotic (LEBEER et al., 2008). All FPA fractions of the samples were able to cause disturbances on survival curves of both bacteria, but the MIC was only observed on *E. coli*. In particular, the FPAs of MR showed a higher microbial-inhibitory action than CP and DR. These results indicated that the application of FPAs of crude propolis and its residues could produce a beneficial selection on the intestinal microbiota. CUEVA et al. (2010) also observed that some *E. coli* strains were highly susceptible to phenolic acids at concentrations of 1 mg/mL or less, but this susceptibility was strain-, structure- and concentration-dependent. Even though *E. coli* is characterized by an outer membrane that provides an intrinsic resistance to antimicrobial compounds, it seems that small phenolic compounds such as phenolic acids could easily cross the membrane and exert their antimicrobial activity (IKIGAI et al., 1993). Moreover, CUEVA et al. (2010) also observed that high concentrations of the same phenolic acids (1 mg/mL) may limit the *in vivo* viability of lactobacilli and their antimicrobial potential might be influenced by the number and positions of substitutions in the phenolic acids' benzene ring. Tabasco et al. (2011) observed a remarkable sensitivity to all commercial phenolic extracts of grape seed on some *Lactobacillus* species, but *L. plantarum*, *L. casei*, and *L. bulgaricus* reached a maximal growth with the phenolic extracts, with some disturbances on the $\mu_{\max}$ depending on the concentration. Meanwhile, *E. coli* strains also reached the maximum growth with all the phenolic extracts, but only a slight reduction on $\mu_{\max}$ were observed at the highest concentration (1 mg/mL).

Unabsorbed dietary polyphenols and their metabolites can behave as activators or inhibitors of bacterial growth depending on their chemical structure and concentration (VIVA et al., 1997; REGUANT et al., 2000). These metabolites can selectively inhibit the pathogen growth and stimulate the growth of commensal bacteria, including some recognized probiotics (CUEVA et al., 2010; LARROSA et al., 2009; LEE et al., 2006), thus influencing the microbiota composition (LAPARRA AND SANZ, 2010). Studies conducted in both humans and animals (e.g. rats, pigs and chickens) reported that the administration of polyphenols from plant sources increases the potential growth of beneficial bacteria such as *Lactobacillus* and decrease the growth of *Enterobacteriaceae*, *Clostridium* and *Bacteroides*, among other bacterial groups (HARA, 1997; DOLARA et al., 2005; MOLAN et al., 2010; VIVEROS et al.,

2011). Therefore, polyphenols can confer health benefits via modulation of the gut microbiota and exert prebiotic-like effects (TZOUNIS et al., 2008; TABASCO et al., 2011). Gut bacteria can also metabolize polyphenols and produce new phenolic compounds “*in situ*”, which could increase their bioavailability and biological activity (REQUENA et al., 2010). Therefore, the possibility of modify selectively the composition of intestinal bacterial species could affect the diversity and metabolic activity of the gut microbiota (CUEVA et al., 2010). This thought has been guiding the search for new compounds that could safe and successfully substitute the use of synthetic antimicrobials in animal feed.

6. CONCLUSIONS

The tetrahydrofuran extraction was efficient to release FPAs from crude organic propolis and its residues. However, the extractions of bound phenolic acids (i.e. SEPA and IEPA) were not effective. The FPA fraction of DR showed higher phenolic content than CP and MR, as well as a higher antioxidant activity in terms of ABTS and DPPH radical scavenging and FRAP. Thus, residues of the ethanolic extraction of propolis still contain some bioactive compounds. FPA fractions of the samples were able to cause disturbances on the growth of *E. coli* and *L. plantarum*, but the MIC was only observed on *E. coli*. In particular, the FPAs of MR showed a higher microbial-inhibitory action than CP and DR. Despite of propolis residues cannot be employed in human nutrition, they could be used in animal feed to modify favorably the gut microbiota.

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GENERAL CONCLUSIONS

The extraction of EOs from organic propolis and its residues presented low yield. The major compounds were α -pinene, β -pinene, and limonene. The EOs exhibited low antioxidant activity and low phenolic content. However, the EOs of propolis and its residues presented a selective action against the pathogenic bacteria *E. coli*. The tetrahydrofuran extraction was efficient to release free phenolic acids (FPA) from crude organic propolis and its residues, however the extraction of bound phenolic acids were not effective. The FPA fractions exhibited antioxidant activity and also were able to cause disturbances on the growth of *E. coli* and *L. plantarum*, but the MIC was only observed on *E. coli*. Thus, residues of the ethanolic extraction of propolis still contain some bioactive compounds. Despite of propolis residues cannot be employed in human nutrition, they could be used in animal feed to modify favorably the gut microbiota.