Chapter 15
Beehives as a Natural Source of Novel Antimicrobials

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Abstract Honey bee products have been used since ancient times as food and therapeutics. There is increasing knowledge on their content and molecular mechanism of action. Their bioactive compounds are a combination of both honey bee and plant origin. Plant immune response effectors are secondary metabolites (polyphenols, terpenes, antimicrobial peptides), and they are responsible for the antimicrobial effects of honey bee products like honey, propolis, and bee pollen. Honey bee innate immunity effectors are antimicrobial peptides, like defensin 1 and 2, apidaecins, abaecins, and hymenoptaecin, and some of them have been found in royal jelly, honey, and pollen. Plant secondary metabolites and honey bee antimicrobial peptides combine in beehive mixtures with synergistic antimicrobial activity and undoubtedly represent an interesting alternative to standard antibiotics. Further research should elucidate their interactions in honey bee products as well as their potential biotechnology applications.

Keywords Honey bees · Honey · Propolis · Royal jelly · Bee pollen · Plant secondary metabolites · Immunity · Antimicrobial peptides

Abbreviations

10-acetooxy-2-DEA	10-Acetoxydecanoic acid
10-HDA	10-Hydroxy-2-decenoic acid
3,10-HDA	3,10-Dihydroxy-decanoic acids
AAMP	Anionic antimicrobial peptides
AMPs	Antimicrobial peptides
CAMP	Cationic antimicrobial peptides
CAPE	Caffeic acid phenethyl ester

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CNS	Coagulase-negative staphylococci
CPPs	Cell-penetrating peptides
CS $\alpha\,\beta$	Cysteine-stabilized α β motif
Cys	Cysteine
Gly	Glycine
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
Imd	Immune deficiency pathway
Jak/STAT	Janus kinase/signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
JV	Junín virus
MAPK	Mitogen-activated protein kinases
MIC	Minimum inhibitory concentration
MRJP	Major royal jelly protein
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NB-LR	Nucleotide-binding leucine-rich repeat containing resistance proteins
NF-κB	Nuclear factor kappa B
P/MAMP	Pathogen/microbe-associated molecular pattern molecules
PR	Pathogenesis-related proteins
Pro	Proline
PRRs	Pattern recognition receptors
RJ	Royal jelly
RLP/Ks	Receptor-like proteins or -kinases
RNAi	RNA interference
RSV	Respiratory syncytial virus
SM	Secondary metabolites
TMV	Tobacco mosaic virus
VRE	Vancomycin-resistant enterococci
VSV	Vesicular stomatitis virus
VZV	Varicella-zoster virus

1 Introduction

In recent decades, as antimicrobial resistance is being increasingly recognized as a global public health threat, natural mixtures with antimicrobial effects such as products from the honey bee *Apis mellifera* are being re-discovered by mainstream medicine.

Beehives have been used as a resource of food and medicines since ancient times. The oldest evidence of humans collecting honey from wild bees dates back to 10,000 years ago (Dams and Dams 1977). Beekeeping started in the early Neolithic period (Roffet-Salque et al. 2016), while according to Crane (1999), domestication of bees was depicted in Egyptian art from around 4500 years ago. Honey was used

in the past in different parts of the world to improve wound and gut healing (Zumla and Lulat 1989). Even the Muslim prophet Mohammed and Aristotle (350 BC) recommended the use of honey for medical purposes (Molan 1999). In Ancient Egypt, propolis was first recognized as an adhesive for sealing cracks in wood, while Aristotle was one of the first to refer to it as a healing agent. In addition, Aristotle was the first to recognize how royal jelly promotes physical strength and intellectual capacity (Fratini et al. 2016a).

Centuries later, with the advent of science, these products have been extensively studied; their composition is analyzed with advanced instrumental methods, while their biological activity is studied in different *in vitro* and *in vivo* assays. As the knowledge about their molecular mechanisms of action grows, we become more aware of their complexities.

The beehive can be viewed as a melting pot of plant and insect defense mechanisms (Fig. 15.1). These defense mechanisms can be extracted in the form of beehive products used as antimicrobial agents. These products are honey, propolis royal jelly, bee pollen, beeswax, and bee venom. Each honey bee product is specific for its content of active compounds, and many of them have a plethora of effects – from antioxidant to antimicrobial.

The compounds vital for plant defense are plant secondary metabolites (SM), abundant in honey bee products. Polyphenols are a huge and versatile group of SM, and many of them can be used as representative markers of honey bee products like propolis. Along with polyphenols, there are terpenoids and plant antimicrobial peptides (AMP). The possible interactions among these compounds yet have to be

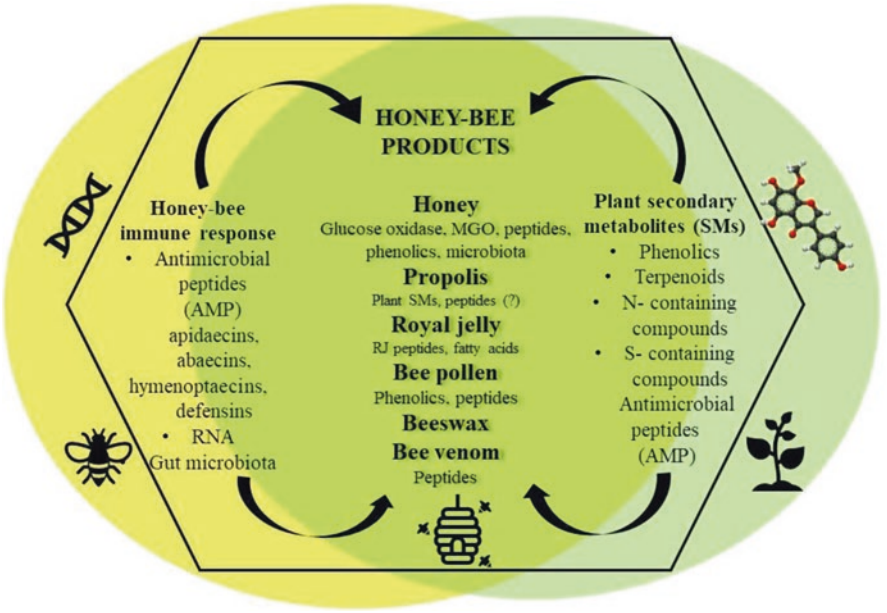

Fig. 15.1 The beehive as the melting-pot of honey bee and plant defense mechanisms

elucidated. Honey bees' defense is based on individual innate immunity and social, collective immunity. Plant and animal material that honey bees integrate into honey bee products is an essential part of the latter. Still, at the same time, these products work through the former – by acting on the intracellular mechanisms vital for individual innate immunity.

In this chapter, I present some of the most relevant antimicrobial compounds that build the defense system of the beehive. These compounds are divided according to their origin, with their role, and antimicrobial effects. Next, honey bee products are described, followed by numerous studies of their antimicrobial efficacy. Undoubtedly, beehives are rich resources of potent antimicrobial compounds, just waiting to be utilized to fight against antimicrobial resistance.

2 Plant Origin of Antimicrobial Substances in the Beehive

Using the beehive as a resource of antimicrobial compounds means considering the immune strategies of insects like honey bees and the vast array of plant–host defense mechanisms. These mechanisms work synergistically as plant, and insect-derived material is combined in honeybee products. Here is where the bees, with all their capabilities, concentrate the abundance of substances from plants and their own, such as polyphenols (flavonoids and phenolic acids), glycoproteins, and antimicrobial peptides, in fighting and resisting various pathogens.

2.1 Plant Immune Response

Plants respond to infection using a two-branched or two-level innate immune system (Jones and Dangl 2006) that needs to be versatile and effective, since plants lack the mobility and a somatic adaptive immune system from animals. The first branch recognizes and responds to molecules common to many classes of microbes, including non-pathogens through defense- receptor-like proteins or -kinases (RLP/Ks) as pattern recognition receptors (PRRs), which can detect conserved pathogen/microbe-associated molecular pattern (P/MAMP) molecules, considered to be an early warning system for the presence of pathogens and the timely activation of plant defense mechanisms (Jones and Dangl 2006; Dubery et al. 2012). A second line of plant defense includes the response to pathogen virulence factors, either directly or through their effects on host targets (Jones and Dangl 2006) via intracellular nucleotide-binding leucine-rich repeat (NB-LR)-containing resistance proteins, which recognize isolate-specific pathogen effectors once the cell wall has been compromised (Dubery et al. 2012).

Proteins and peptides involved in these mechanisms can be found in plant material collected by honey bees and integrated in honey and royal jelly products. One of the most studied antimicrobial peptides, defensins, found in bees, honey, and royal jelly could be partly of plant origin. Furthermore, plant polyphenols are highly

versatile secondary plant metabolites, allowing plants to respond promptly to unpredictable stress agents of different origins (Wink 2008).

2.2 Plant Secondary Metabolites (SMs)

General resistance in plants is achieved by the production of secondary metabolites (SMs), a highly diverse group of organic molecules which are not necessary for the actual metabolism or physiology of the plants producing them. These compounds serve as protective agents against various pathogens: bacteria, fungi, viruses, and insects (Wink 2008). There are several different classes of SMs: phenolic compounds (flavonoids, tannins), terpenoids, N-containing compounds (non-protein amino acids, cyanogenic glucosides alkaloids), and S-containing compounds (pathogenesis-related (PR) proteins, phytoalexins) (Wink 2008; Jamwal et al. 2018). In nature, these metabolites always come in complex mixtures.

Polyphenols

One of the most abundant groups of SMs in honey bee products is polyphenols. Polyphenols can be divided into several classes: flavonols, flavones, flavanones, anthocyanidins, flavanols, and isoflavones (Daglia 2012). Polyphenols were studied mostly because of their antioxidant effect as the basis for chronic disease prevention, but with the increase of antimicrobial resistance, their antimicrobial potential came into focus as well.

In general, flavonoids have shown stronger antimicrobial activity than non- flavonoid compounds. Flavan-3-ols, flavonols, and tannins were extensively studied due to their wide spectrum and higher antimicrobial activity compared to other polyphenols. Most of them can suppress many microbial virulence factors (such as inhibition of biofilm formation, reduction of host ligands adhesion, and neutralization of bacterial toxins) and show synergism with antibiotics (Daglia 2012). Although weaker than flavonoids, non-flavonoids such as phenolic acids (caffeic and ferulic acids) showed activity against Gram-positive (*Staphylococcus aureus, Listeria monocytogenes*) and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) (Daglia 2012).

There are several mechanisms of polyphenol antimicrobial activity (Olchowik-Grabarek et al. 2020): the damage of the cell membrane and cell wall (Funatogawa et al. 2004; Yi et al. 2010; Adnan et al. 2017), inhibition of energy metabolism (Li et al. 2017), production, secretion, structure, and activity of released toxins (Hisano et al. 2003; Shah et al. 2008; Lee et al. 2012; Dong et al. 2013; Verhelst et al. 2013; Wang et al. 2015; Song et al. 2016; Shimamura et al. 2015; Chang et al. 2019; Tang et al. 2019) and biofilm formation (Lin et al. 2011; Trentin et al. 2013). Polyphenols also act at the level of target cells, increasing their resistance to toxins (Olchowik-Grabarek et al. 2020). Regarding polyphenol interaction with cell structures, it was

hypothesized that the polyphenols rich in gallate moieties might attach to the cell surface, serve as bridges between surfaces of two neighbor cells, and initiate cell-binding and formation of similar clusters in the membrane of the opposite cell (Tarahovsky 2008). Phan et al. (2014) confirmed that an increase in the number of hydrophilic side chains (galloyl, hydroxyl, glucoside, gallate) increased the reactivity of the polyphenols with cell membranes. Due to their polarity, they are not able to pass the cell membrane through passive diffusion, so it is assumed that they pass through the membranes with the help of other plant SMs (Wink 2008).

The interactions of polyphenols with proteins and peptides are interesting, not only for a better understanding of their action on cell surfaces and signal transduction pathways but to understand how these molecules will interact with each other in a natural mixture like those found in the beehive. Peptides and polyphenols form noncovalent (hydrogen, hydrophobic, and ionic bonds) and covalent bonds (between oxidized phenolic compounds and peptides) (Sun and Udenigwe 2020).

While forming ionic bonds, negatively charged phenolate ions interact with positively charged amino acids. Depending on their size, a single polyphenol can bind even several proteins simultaneously (Wink 2008). Bourvellec and Renard (2012) describe how, at the same time when hydrophobic bonds form between polyphenol aromatic rings and hydrophobic residues of amino acids, hydrogen bonds are also formed between the hydroxyl groups of polyphenols and the acceptor site for hydrogen ions in the proteins (Bourvellec and Renard 2012). The primary factors affecting the protein–polyphenol interaction are conformation and type of both proteins and polyphenols. Other factors are environmental conditions, like temperature and pH (Quan et al. 2019). It is assumed that the phenolic binding can affect protein activity or even protect proteins from proteolytic cleavage (Wink 2008). When polyphenols oxidize to reactive quinones, they form covalent bonds with proteins in honey, and this complexation can lead to decreased antioxidant, enzymatic, or antimicrobial activity (Brudzynski and Maldonado-Alvarez 2015).

On the other hand, there is growing evidence that the formation of protein/peptide conjugates results in increased antioxidant activity and stability in food (Quan et al. 2019). Possibly, the same logic could be applied to their antimicrobial activity, and we assume that polyphenols could increase the stability and the activity of antimicrobial peptides.

Volatile SMs (Terpenoids)

The volatile SMs necessary for plant defense are complex mixtures of hydrocarbons and oxygenated hydrocarbons from the isoprenoid pathways, primarily monoterpenes and sesquiterpenes (Bankova et al. 2014). They are produced and secreted by glandular trichomes; specialized secretory tissues diffused onto the surface of plant organs, particularly flowers and leaves (Bankova et al. 2014).

Plant Antimicrobial Peptides (AMPs)

Plants produce PR proteins/peptides with numerous defense-related properties, including antibacterial, antifungal, antiviral, antioxidative activity, chitinase, and proteinase inhibitory activities (Tam et al. 2015). Antimicrobial peptides (AMPs) interact with cell membrane phospholipids and cell-penetrating peptides (CPPs), which introduce certain cargoes in the cell (Nawrot et al. 2014).

AMPs have been isolated from all parts of plants and can be divided into anionic (AAMPs) and cationic (CAMPs) peptides. These groups have shown activity against pathogenic microorganisms (bacteria, viruses, and fungi) and even neoplastic cells (Montesinos 2007; Nawrot et al. 2014). Antimicrobial peptides (AMP) found in plants are rich in Cys, enabling disulfide bonds. This contributes to their stability and resistance to enzymatic degradation. (Tam et al. 2015). According to Nawrot et al. (2014), there are six groups of plant AMPs: thionins, defensins, lipid transfer proteins, cyclotides, hevein-like proteins, and knottin-type proteins.

AMPs mechanism of antimicrobial action has been described through several types of models of membrane pore formation, which leads to cell content leakage and death. AMPs act on the microorganism cell membrane due to their negative charge, which attracts cationic peptides. In the bacterial membrane, negatively charged molecules, and thus main receptors of CAMPs are phospholipids. While in fungal membranes, these are glucosylceramides and sphingolipids. In addition, many CAMPs appear to target internal anionic cell constituents, such as DNA, RNA, or cell wall components (Diamond et al. 2009). AMPs exhibit broad-spectrum activity, and thus far, it appears as though bacteria do not develop resistance as quickly as with conventional antibiotics (Diamond et al. 2009).

While the mechanisms of CAMPs are better understood, those of AAMPs are less so. There is evidence suggesting they increase plasma membrane permeability by binding to lipids, disrupting the envelope integrity by attaching to chitin, and damaging intracellular structures, such as DNA. It is also proposed that AAMPs participate in the plant innate immune response and act synergistically with CAMPs (Prabhu et al. 2013). Prabhu et al. (2013) conclude that cyclotides are the plant AAMPs with the greatest potential for therapeutic and biotechnical development. Cyclotides are named after the cyclic peptide backbone and a knotted arrangement of three conserved disulfide bonds. Due to those bonds, they are relatively stable to thermal, chemical, and enzymatic degradation and can be modified by residue substitutions (Prabhu et al. 2013). One of the best-studied cyclotides, kalata B2, was found to have potent antibacterial activity against *Salmonella enterica, E. coli,* and *S. aureus* (Gran et al. 2008; Pranting et al. 2010), but also against parasites like gastrointestinal nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis* (Colgrave et al. 2008). Other known antimicrobial cyclotides with antibacterial activity are vaby D (Pranting et al. 2010) and cycloviolacin O24 (Ireland et al. 2006) and cycloviolacins Y1, Y4, and Y5 which exhibit anthelmintic properties (Colgrave et al. 2008) and antiviral activity (Wang et al. 2008).

The two most prominent plant CAMP families are thionins and defensins. There are several common traits of these two CAMP families between various species (microbes, plants, animals), and those include their amphipathic nature, positive charge, and molecular structure. These peptides are membrane-active, while other families of AMPs have a different mechanism of action – from enzyme inhibition to lipid transfer. Thionins are AMPs with a small molecular weight (~5 kDa) rich in arginine, lysine, and cysteine residues (Nawrot et al. 2014). There are two groups of thionins, α−/β- and γ-thionins (based on their structure, γ-thionins are considered to be a part of the defensin family of peptides). They are toxic against phytopathogenic bacteria, fungi (Ebrahimnesbat et al. 1989), and yeasts, and also some animal and plant cells (Evans et al. 1989). They interact with the protein receptors or lipids in membranes (Osorio e Castro and Vernon 1989; Florack and Stiekema 1994; Garcia-Olmedo et al. 1998; Stec 2006) with their hydrophobic residues and positive surface charge to cause cell leakage and lysis (Majewski and Stec 2001; Tam et al. 2015). Thionins isolated from black seed (*Nigella sativa*) showed bactericidal and fungicidal effects on *Bacillus subtilis, S. aureus,* and *Candida albicans* (Vasilchenko et al. 2017).

Defensins are well-known and abundant AMP in plants, vertebrates, and invertebrate animals (Nawrot et al. 2014; Tam et al. 2015) and fungi (Wu et al. 2014). They are also of small molecular weight (~5 kDa), cysteine rich and cationic peptides with broad-spectrum antimicrobial activity; antibacterial, antifungal, antiviral, proteinase, and insect amylase inhibitor (Nawrot et al. 2014). Their previously described mechanisms of antimicrobial activity are based on membrane lysis. Still, there are other processes by which they disrupt, such as interfering with cell signaling, intracellular trafficking, blocking the receptor binding, and cell entry (Weber 2020). Plant defensins are ancient and conserved; therefore, they are similar to honey bees and vertebrate animals (Nawrot et al. 2014). They also act as immunomodulators by attracting immune cells and modulating adaptive immune responses (Weber 2020).

Despite having only identified and isolated AMPs from honey bees and their products, one cannot exclude the possibility that some of these peptides are of plant origin since there is a certain amount of plant material in the beehive. One cannot also exclude the possible relevance of these peptides, such as in the case of polyphenols and other secondary plant metabolites that have been identified in honey, pollen, or propolis.

3 Honey Bee Defense Mechanisms

Honey bees are social insects with a collective “social immunity” and an individual innate immunity, which consists of humoral and cellular effectors (Evans et al. 2006).

3.1 Honey Bee Individual Immunity

Cells involved in individual honey bee immune response are phagocytes and hemocytes and humoral-induced effectors such as AMPs, thioester linkage proteins, melanization, and coagulation proteins (Larsen et al. 2019). Antiviral intracellular defense mechanisms include RNA interference (RNAi), endocytosis, melanization, encapsulation, autophagy, and conserved immune pathways including Jak/STAT (Janus kinase/signal transducer and activator of transcription), JNK (c-Jun N-terminal kinase), MAPK (mitogen-activated protein kinases), and the NF-κB mediated Toll and Imd (immune deficiency) pathways (McMenamin et al. 2018). Interestingly, RNAi is the key resistance mechanism against viruses, not only for individual honey bees but also for the whole beehive's immune response (Maori et al. 2019). Similarly, Toll, Imd, Janus kinase (JAK)/STAT, and JNK are signaling pathways induced by bacterial cell wall lipopolysaccharides or peptidoglycans (Boutros 2002; Evans et al. 2006) and result in the release of antimicrobial effectors, peptides, such as hymenoptaecin, defensin 1, and abaecin at the end of the cascade (Evans et al. 2006; Gätschenberger et al. 2013). As in plants, AMPs are considered the key component of honey bee innate immunity (Danihlík et al. 2015).

3.2 Honey Bee AMPs

Both honey bee products and antimicrobial peptides (AMPs) have been recognized as resources of promising alternatives to conventional antibiotics. AMPs have been described as ancient evolutionary weapons produced by many living organisms as a part of their nonspecific immune response. Thus, they are effective against many microorganisms (Baltzer and Brown 2011). AMPs exhibit a multimodal mechanism of action, specifically responding to various intracellular targets and binding to lipopolysaccharides of the bacterial membrane with different, concentration-dependent affinity (Baltzer and Brown 2011; Hughes et al. 2000; Li et al. 2012).

As plant AMPs, insect AMPs form pores on the cell membrane of bacteria in different ways (Li et al. 2012). They can also bind to different intracellular targets (DNA, RNA, and proteins) once inside the cell and inhibit their synthesis (Lan et al. 2010; Li et al. 2012). Moreover, insect AMPs can interfere with bacterial cytokinesis by cell filamentation, using unique translocation mechanisms to alter the cytoplasmic membrane septum formation (Brown and Hancock 2006; Lan et al. 2010; Li et al. 2012).

Not only do they have broad-spectrum activity against microorganisms, but AMPs are also able to bypass the common resistance mechanisms that render conventional antibiotics ineffective (Wang et al. 2016). Apart from antimicrobial activity, AMPs also modulate the immune system via cytokine activity or angiogenesis (Li et al. 2012). Potential novel therapeutics such as AMPs could be

implemented using natural mixtures that may have antimicrobial and immunomodulatory activity due to their complexity and molecular synergism.

Based on their structure, insect AMPs can be divided into four categories: α-helix (cecropin and moricin), Cys-rich (insect defensin and drosomycin), Pro-rich (apidaecin, drosocin, and lebocin), and Gly-rich peptides (attacin) (Bulet and Stöcklin 2005; Yi et al. 2014). Honey bees pathogens induce four families of AMPs; apidaecins, abaecins, hymenoptaecins, and defensins. These families have a broad spectrum of antimicrobial activity in the hemolymph (Xu et al. 2009). Besides the active AMPS in adult honey bee lymph, inactive peptide precursors can be found in bee larvae (Casteels et al. 1989). Apidaecins were found to be very selective and active against human and animal Gram-negative bacteria (*E. coli*, *Salmonella,* and *Shigella* species) (Casteels et al. 1989), while abaecins are more active against Gram-positive bacteria (Casteels et al. 1990). To be more specific, in comparison to abaecins, apidaecins showed 200-fold more activity against *Agrobacterium*, *Erwinia,* and *E. coli* strains (Casteels et al. 1990). In the same study, abaecins showed the highest specific activity against plant pathogen *Xanthomonas campestris*. This was expected since honey bees are often exposed to plant-associated microorganisms whilst gathering food, pollen, and nectar. Hymenoptaecin is active against Gram-negative and Gram-positive bacteria, including several human pathogens (Casteels et al. 1993). Its bactericidal effect against *E. coli* results from sequential permeabilization of the outer and inner membranes (Casteels et al. 1993). When combined in immune lymph, hymenoptaecin, and apidaecin, as the two predominant factors, had a strong bactericidal effect against a broad spectrum of Gram-negative (*Bordetella bronchiseptica, Enterobacter cloacae, Haemophilus influenzae, Yersinia enterocolitica,* etc.) and some Gram-positive bacteria. Defensins killed Gram-positive bacteria (e.g., Clostridium and Streptococcus species) that were unaffected by their combination. As Casteels et al. (1993) concluded, "it is clear that the broad-spectrum antibacterial activity of immune lymph is the result of an amazing complementarity."

As in plants, defensins are the most abundant group of AMPs in insects. In general, insect defensins have an N-terminal loop and an α-helical fragment followed by an antiparallel β-structure, connected by two of the three disulfide bridges. These form so-called cysteine-stabilized $\alpha\,\beta$ (CS $\alpha\,\beta$) motif (Cornet et al. 1995). Defensins have antibacterial activity against Gram-positive bacteria, including *S. aureus*, *Micrococcus luteus*, and *Aerococcus viridans* (Yi et al. 2014; Li et al. 2017). Two types of defensins have been identified in honey bees. Defensin 1 is synthesized in salivary glands and plays an important role in social immunity, while defensin 2 is synthesized by cells of body fat and lymph, which is an important factor in the system of the honey bee individual immunity (Ilyasov et al. 2013).

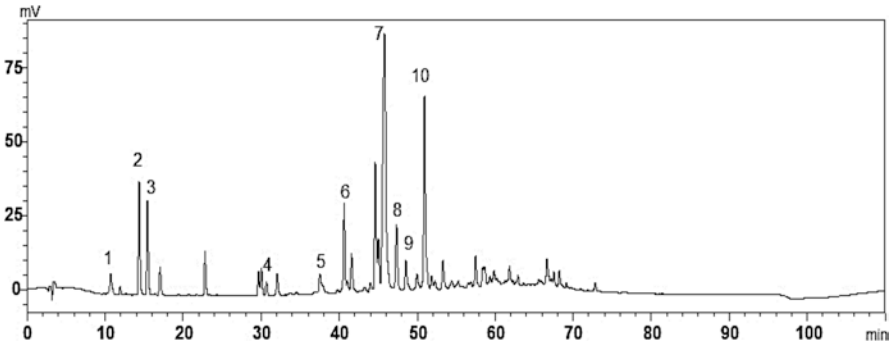

Fig. 15.2 The typical HPLC-UV chromatogram of propolis extracts obtained in our laboratory. Ten biomarkers are used for analysis: (1) caffeic acid, (2) p-coumaric acid, (3) ferulic acid, (4) trans-cinnamic acid, (5) kaempferol, (6) apigenin, (7) chrysin, (8) pinocembrin, (9) CAPE, (10) galangin

3.3 Honey Bee Social Immunity

Honey bees use social immunity as a collective defense against pathogens (DeGrandi-Hoffman and Chen 2015). This type of response is based on behavioral cooperation (Evans and Spivak 2010) during small tasks that have a colony-wide impact on reducing pathogenic invasion, for example, necrophoric and hygienic behavior (removing the dead adults or diseased brood from the colony), or thermoregulatory activity (workers produce high temperature) against heat-sensitive pathogens (DeGrandi-Hoffman and Chen 2015). The previously mentioned transmissible RNA pathway through the royal jelly and worker jelly also has an important role in social immunity and signaling between hive members. It protects bees against viruses and the *Varroa* mite (Maori et al. 2019).

Nutrition is a key factor in honey bees' social and individual immunity (DeGrandi-Hoffman and Chen 2015). Honey bees use plants as their food but also as a form of their external, collective immunity. Bee pollen is a primary source of food for the beehive, entirely of plant origin. Honey is produced partly from the sugary secretions of plants (floral nectar). The most effective honey bee product with immunomodulatory, antimicrobial, antioxidative activity is propolis. Propolis is a resin derived from plants combined with animal origin substances – honey bee saliva and beeswax – rich in polyphenols from plants (Bankova et al., 2021). These polyphenols are used as markers of the biological activity of propolis (Fig. 15.2).

As previously mentioned, to protect themselves against consumption by herbivores and pathogens, plants use complex mixtures of numerous secondary compounds (SM) (Wink 2008). The action of these compounds in mixtures can be synergistic or antagonistic. Mechanisms of activity are pleiotropic and interact with many targets at the same time. As such, these compounds have many advantages

over mono-target compounds (Wink 2008). Some common mechanisms include modulation of the structure and function of proteins, interference with gene expression, and changing membrane permeability. Most of these SMs have been found in the beehive in honey bee products.

4 Honey Bee Products as Beehive Defense Resources

There are six main products from the beehive with antimicrobial effect described in the scientific literature: honey, propolis, royal jelly, pollen, beeswax, and bee venom. Of these, honey and propolis antimicrobial activities have been studied the most and have the greatest potential in treating systemic or local infectious diseases.

4.1 Honey

The first product from the beehive used for its antimicrobial properties (besides the nutritional) in folk medicine was honey. Honey is the end product of nectar digestion and is stored in honeycomb cells. In terms of content, honey is made up of a supersaturated aqueous solution. This solution is comprised of 80% sugars, mostly fructose, and glucose.

It is known that natural unheated honey has some broad-spectrum antibacterial activity when tested against methicillin-resistant *S. aureus* (MRSA), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, vancomycin-resistant enterococci (VRE), extended-spectrum β-lactamase-producing (ESBL) *Proteus mirabilis*, and *E. coli*. There are numerous studies on the antimicrobial activity of different types of honey. In one study, the MICs of Tualang honey ranged 8.75%–25% compared with those of manuka honey (8.75%–20%) against the wound and enteric microorganisms: *S. pyogenes,* CNS, MRSA*, Streptococcus agalactiae, S. aureus, Stenotrophomonas maltophilia, Acinetobacter baumannii*, *S. enterica* Serovar *typhi, P. aeruginosa, P. mirabilis, Shigella flexneri, E. coli,* and *E. cloacae* (Tan et al. 2009). In time-kill studies, antibiotic susceptible and resistant isolates of *S. aureus, S. epidermidis, Enterococcus faecium, E. coli, P. aeruginosa, E. cloacae,* and *Klebsiella oxytoca* were killed within 24 h by 10–40% (v/v) honey (Mandal and Mandal 2011). Several types of honey were tested against planktonic and biofilm-grown bacteria and showed 100% bactericidal efficacy against planktonic forms. The bactericidal rates for the Sidr and two types of Manuka honey against MSSA, MRSA, and *P. aeruginosa* biofilms were 63–82%, 73–63%, and 91–91%, respectively (Alandejani et al. 2009).

Different types of honey also displayed specific antiviral effects. Manuka and clover honey showed activity against varicella-zoster virus (VZV) in concentrations ranging from 0% to 6% wt/vol (Shahzad and Cohrs 2012). In addition, a randomized controlled trial on the efficacy of honey compared to acyclovir showed comparable

success rates of topical application of medical-grade kanuka honey and 5% aciclovir in the treatment of herpes labialis (Semprini et al. 2019).

These antimicrobial effects are attributed to a wide array of compounds found in honey, such as oligosaccharides (Cornara et al. 2017), glucose oxidase, and non-peroxide factors with antibacterial activity, like methyl syringate, methylglyoxal (MGO), peptides from honey bees (defensin-1) (Cornara et al. 2017), and honey glycoproteins (glps). Honey glycoproteins showed sequence identity with the major royal jelly proteins 1 (MRJP1) precursor (Brudzynski and Sjaarda 2015), and also the concentration-dependent antibacterial activity against Gram-positive *Bacillus subtilis* and Gram-negative *E. coli*. These glycoproteins bind and agglutinate bacterial cells and also cause membrane permeabilization (Brudzynski and Sjaarda 2015). Glucose oxidase is added by bees, which, by low dilution, converts glucose into H_2O_2 and gluconic acid.

Active compounds of plant origin that are found in honey differ based on the botanical origin of their néctar. Some types of honey are being marketed as specific regarding their antimicrobial effects and so-called unique factors. What they all have in common is supersaturation (high osmolarity, osmotic effect), low water activity, and low pH. These factors cultivate an unfavorable environment for microbial growth (Tan et al. 2009).

Microbiota from honey is also believed to be responsible for its antibacterial activity. Fourteen bacterial isolates of *Bacillus* sp. showed antimicrobial activity against *C. albicans*, *E. coli*, and *S. aureus* has been found in honey (Jia et al. 2020).

4.2 Propolis

Honey bees primarily use propolis as a construction material but also to maintain beehive health. Propolis is also used as an important part of social immunity due to its natural antiseptic properties (Bankova et al., 2018; Bankova et al., 2021). It is a resinous mixture of both animal and plant origin—bees collect it from exudates and plant buds, where it is further mixed with wax and saliva enzymes (Bankova et al., 2021). Its chemical composition varies depending on the geographical and botanical origin: the most common type of propolis in Europe is poplar-type, from *Populus nigra*. The most prevalent types of Brazilian propolis are green due to plant *Baccharis dracunculifolia* and red, from plant *Dalbergia ecastophyllum.* Brown Cuban propolis, the principal type of Cuban propolis, is derived from *Clusia rosea*. Each type of propolis contains about 300 bioactive compounds (Sforcin and Bankova 2011; Pellati et al. 2013); triterpenes (50% w/w), waxes (25–30%), volatile mono- and sesquiterpenes (8–12%) and phenolics (5–10%) (Huang et al. 2014).

Most active compounds are of plant origin and are believed to be responsible for the antimicrobial, antioxidant, immunomodulatory, and anti-inflammatory activities of propolis (Sforcin and Bankova 2011). The antimicrobial activity of propolis was confirmed when tested against bacteria, viruses, yeasts, and even parasites. Propolis extracts are highly active against Gram-positive (MRSA, VRE, *Streptococcus*

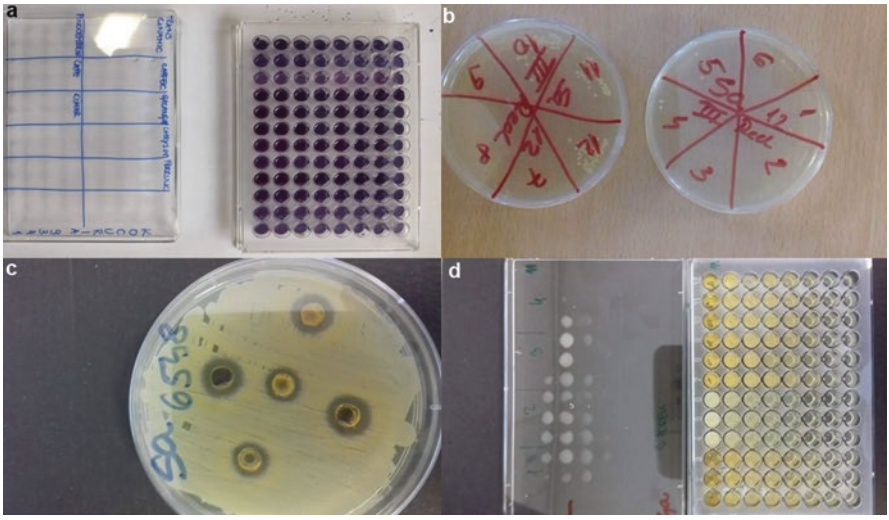

Fig. 15.3 Antimicrobial susceptibility testing: minimal biofilm eradication concentration (MBEC) determination for different (separate) propolis biomarkers (**a**), and propolis extracts minimum inhibitory concentrations (MICs) determination by subcultivation on agar plates (**b**), agar well diffusion (**c**), and broth microdilution (**d**) method. (With courtesy of Dr. Josipa Vlainić)

species, *B. subtilis, S. aureus, Enterococcus faecalis*) and less active against Gram-negative bacteria like *E. coli.* However, they have bactericidal activity on *P. aeruginosa* (Kosalec et al. 2005, Przybyłek and Karpin´ski 2019). Propolis is also active against yeasts like *Candida* species (Kosalec et al. 2005) and many viruses *in vitro* and *in vivo* (Berretta et al. 2020; Nolkemper et al. 2010; Schnitzler et al. 2010). The mechanism of action depends on inhibition of the virus' entry into cells and disruption of viral replication, which destroys RNA before or after its release in the cells (Búfalo et al. 2009; Sforcin 2016). Propolis components have inhibitory effects on the ACE2, TMPRSS2, and PAK1 signaling pathways and can potentially interfere with the host cell invasion by SARS-CoV-2 (Berretta et al. 2020).

It is presumed that the antimicrobial activity depends on the presence of flavonoids such as galangin, pinocembrin, rutin, quercetin, naringenin, and CAPE, since these compounds are known to increase bacterial membrane permeability. Some of those compounds (galangin, pinocembrin, CAPE) also inhibit bacterial RNA polymerase (Cornara et al. 2017). It is, therefore, clear that the antimicrobial activity of propolis is a result of the mixture effect and synergy between the flavonoid compounds and that the resultant antimicrobial actions are understood so far as complex mechanisms. Due to this complexity, propolis is active against multidrug-resistant bacteria (Pamplona-Zomenhan et al. 2011; Przybyłek and Karpin´ski 2019).

We confirmed this synergy when we compared the MIC values of propolis extracts with different amounts of active markers (p-coumaric acid, trans-ferulic acid, caffeic acid, CAPE, cinnamic acid, chrysin, pinocembrin, galangin, apigenin, kaempferol) (Fig. 15.3).

An interesting and completely unexpected result is that the mixture of these active substances in small concentrations is more effective than that of much higher concentrations of certain (pure) active substances alone (work in progress) (Fig. 15.3). It seems that the synergy effect between these compounds follows the Goldilocks principle.

There are certainly other compounds relevant to the investigation of propolis-mediated antimicrobial activity. These may not just be of plant, but honey bee origin, such as antimicrobial peptides found in other honey bee products. Based on the previously posited interaction pathways between peptides and polyphenols (Wink 2008; Quan et al. 2019), peptides in propolis could exert great stability and possibly enhanced therapeutic potential.

Surprisingly, the idea of propolis as a natural source of stabile AMPs has never been tested before. Our preliminary and currently ongoing research confirmed peptides like MRJP1 and some peptides related to *Populus* genus in raw propolis samples. There remains a wealth of other detected peptides yet to be sequenced.

4.3 Royal Jelly as a Resource of Antimicrobials

Royal jelly (RJ) is a food for all bee larvae for the first 3 days of their life. For the queen bee, RJ serves as the source of all subsequent nutrition throughout her lifespan. RJ is a white-yellow, colloidal, slightly acidic secretion produced from the hypopharyngeal and mandibular salivary glands of young bees (nurse, aged between 5 and 14 days) (Fujita et al. 2013; Fratini et al. 2016a). It consists of 60–70% water, 11%–23% carbohydrates, 9–18% proteins, 4–8% lipids, and the remaining 0.8–3% are vitamins, minerals, and even phenolic compounds, presumably from plants (Sabatini et al. 2009; Fratini et al. 2016a). The composition varies based on the season and nutrition of the bees.

Bioactive peptides and proteins identified in royal jelly are the families of major royal jelly proteins (MRJPs), royalisin, glycoproteins jelleins, apolipophorin III-like protein, glucose oxidase (Fratini et al. 2016a), defensin, apidaecins and hymenoptaecin (Han et al. 2014). Interesting components of royal jelly with antibacterial activity are unsaturated fatty acids, such as 10-hydroxy-2-decenoic (10-HDA), also known as queen-bee acid (Fratini et al. 2016a).

MRJPs have a significant role in honey bee nutrition since they account for 82–90% of total larval jelly proteins and contain essential amino acids. There are seven members of the MRJP family (MRJP 1–7) that have health-promoting effects and two members without these healthful advantages (Ahmad et al. 2020). MRJP1 occurs as a monomer (mono MRJP1 or royalactin), or can also appear as an oligomer known as apisin, when polymerized with apisimin (Ahmad et al. 2020). MRJP1 has been shown to modulate biological function in a broad range of species and can maintain pluripotency by activating a ground-state pluripotency-like gene network (Wan et al., 2018). However, it seems that MRJP1 does not display specific antimicrobial properties (Bucekova and Majtan 2016).

Nevertheless, jelleins, peptides isolated from MRJP1, showed a broad spectrum of activity against Gram-positive (*B. subtilis, S. aureus, Paenibacillus larvae*), Gram-negative bacteria (*E. coli, P. aeruginosa*), and against *C. albicans.* The MICs of synthetic jelleins varied between 2.5 μg/ml against *E. coli* and 15 μg/ml against *S. saprophyticus* (Brudzynski and Sjaarda 2015). Jellein I and Jellein II were active against *S. aureus*, *Staphylococcus saprophyticus,* and *B. subtilis* among the Gram-positive bacteria, and *E. coli*, *Enterobacter cloacae*, *K. pneumoniae,* and *P. aeruginosa* among the Gram-negative bacteria (Romanelli et al. 2011). Jellein III showed a narrower spectrum of general activity (Romanelli et al. 2011) but was the strongest in reacting against *S. epidermidis* (Cappareli et al. 2012).

MRJP2 and MRJP4 act as antimicrobial agents and have a wide range of activity against bacteria (Gram-positive and Gram-negative), fungi, and yeasts (Ahmad et al. 2020). They kill microorganisms by attaching to, and damaging, the cell wall of fungi, yeast, and bacteria (Kim et al. 2019; Park et al. 2019).

Royalisin is strongly active against Gram-positive bacteria strains of *Bifidobacterium, Clostridium, Corynebacterium, Lactobacillus, Leuconostoc, Staphylococcus,* and *Streptococcus* genera, with inhibitory efficacy comparable to that of antibiotics (Fratini et al. 2016a). Apolipophorin-III-like proteins (lipid transport proteins) and phosphorylated icarapin (venom protein-II) are the components of royal jelly that promote immune response (Ahmad et al. 2020).

The antifungal properties of royal jelly are not limited only to their peptide properties but can also be attributed to fatty acids, such as 3,10-HDA, 10-HDA, and 10-acetooxy-2-DEA, that inhibit the growth of *Candida tropicalis*, *C. albicans*, and *Candida glabrata* (Meliou and Chinou 2005).

Antiviral effects of royal jelly are not attributed to certain peptides but to the product as a whole. Honey, royal jelly, and acyclovir have the highest inhibitory effects on HSV-1 at concentrations of 500, 250, and 100 μg/mL, respectively (Hashemipour et al. 2014).

4.4 Honey Bee Pollen

Honey bee pollen is used as a raw material to produce bee bread. Bee bread is the main protein source for the bee colony and the source of nutritional and mineral substances for royal jelly produced by worker bees (Komosinska – Vassev et al. 2015). Pollen is also important for the production and expression of antimicrobial peptides—apidaecins and abaecin—in honey bees, not just due to its microbiota, but possibly to certain immunomodulatory protein factors that yet have to be determined (Danihlík et al. 2018).

Honey bee pollen composition varies depending on the botanical and geographical origin of the pollen grains. Generally, pollen consists of proteins, amino acids, carbohydrates, lipids, fatty acids, phenolic compounds, enzymes, and coenzymes, and vitamins and elements. There are approximately 200 substances from different plant species found in pollen grains (Komosinska – Vassev et al. 2015). It is believed

that plant SMs like flavonoids and phenolic acids are responsible for pollen antioxidant and antimicrobial activity (Bridi et al. 2019). These effects are also possibly mediated by glucose oxidase activity, deriving from honey bee secretion (Cornara et al. 2017).

Bee pollen extract showed antibacterial activity against Gram-positive bacteria like *Streptococcus pyogenes* (Bridi et al. 2019), *S. aureus*, Gram-negative bacteria, including *E. coli, K. pneumoniae, Pseudomonas aeruginosa,* and on fungi such as *C. albicans* (Komosinska – Vassev et al. 2015).

Bee pollen is a component of honey and propolis and, as such, adds to their antimicrobial efficacy. When compared by their pollen content, heterofloral honey samples from Turkey, with pollen dominantly from *Chenopodiaceae/Amaranthaceae*, *Trifolium, Trigonella, Cyperaceae, Zea mays,* and *Anthemis* taxa, had the highest antibacterial activity against *P. aeruginosa, E. coli,* and *S. aureus* (Mercan et al. 2007). However, in our MIC study on Gram-positive and Gram-negative bacteria, we found no bactericidal or bacteriostatic activity of *Cistus* pollen extracts.

4.5 Beeswax

Honey bees secrete beeswax in order to build honeycombs. Beeswax is a complex mixture (more than 300 components) of hydrocarbons, free fatty acids, esters of fatty acids and a fatty alcohol, diesters, and exogenous substances (Tulloch, 1980), which are mainly residues of propolis, pollen, small pieces of floral component factors, and pollution (Hepburn et al. 1991).

Several studies report antimicrobial activity of crude beeswax against *S. aureus, Staphylococcus epidermidis, Streptococcus pyogenes*, *B. subtilis, P. aeruginosa, E. coli, S. enterica, C. albicans,* and *Aspergillus niger* (Fratini et al. 2016b). Similarly, beeswax methanolic and ethanolic extracts showed inhibitory activity on *L. monocytogenes, S. enterica*, *E. coli*, *A. niger, C. tropicalis, C. glabrata,* and *C. albicans* (Fratini et al. 2016b).

Beeswax also has good antimicrobial activity in synergy with other natural products, like propolis, honey, or olive oil (Fratini et al. 2016b).

4.6 Bee Venom (Apitoxin)

Honey bee venom glands secrete the venom and inject it through a stinger. Bee venom is rich in amphipathic polycationic peptides, melittin and apamin, enzymes such as phospholipase A2, and low-molecular-weight compounds including active bioamines such as histamine and catecholamines (Cornara et al. 2017). This complex mixture causes local inflammation, anticoagulant effect, and immune response in victims (Cornara et al. 2017).

Melittin, a peptide of 26 amino acid residues, has been recognized as a peptide with an antiviral effect. It has inhibited the viral replication of *Herpes simplex* virus (HSV), human immunodeficiency virus-1 (HIV-1), and Junín virus (JV), and it also has shown to reduce the infectivity of *Coxsackie* virus and other enteroviruses (*Picornaviridae*), Influenza A viruses (*Orthomyxoviridae*), respiratory syncytial virus (RSV; *Pneumoviridae*), vesicular stomatitis virus (VSV; *Rhabdoviridae*), and the plant virus tobacco mosaic virus (TMV; *Virgaviridae*) (Memariani et al. 2020). Melittin also showed effective antibacterial activity against *Streptococcus salivarius, Streptococcus sobrinus, Streptococcus mutans, Streptococcus mitis, Streptococcus sanguinis, Lactobacillus casei,* and *E. faecalis* with MIC values ranging from 4 to 40 μg/mL (Leandro et al. 2015). Although melittin has many therapeutic potentials, the systematic administration is followed by many side effects, and its biotechnological applications are limited to topical formulations (Moreno and Giralt 2015).

5 Conclusion

Honey bee products result from combining the honey bee and plant-origin compounds in the beehive, and as such, have been used as food and therapeutics since ancient times. They are abundant in sugars, secondary plant metabolites, and honey bee proteins and peptides with antimicrobial activity. With the help of powerful modern technologies stemming from molecular biology, proteomics, and chemistry, the evidence and mechanisms of their antimicrobial activity are being elucidated increasingly. However, one must bear in mind the effect of the mixture and synergy between the components in natural products.

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