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Original article

Efficacy of manuka honey with conventional antifungals on *Malassezia pachydermatis*

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Abstract

Yeast infections such as otitis externa and seborrheic dermatitis in dogs and cats are frequently associated with *Malassezia pachydermatis* secondary infection. It is part of the normal cutaneous microflora of most warm-blooded vertebrates, however, under certain conditions, it can become a causative agent of infection that needs to be treated pharmacologically. Azole derivatives are the drugs of the first choice. An interesting trend in developing resistance is the use of natural substances, which include manuka honey with confirmed antimicrobial properties. The main intention of this research was to evaluate the mutual effect of manuka honey in combination with four conventional azole antifungals – clotrimazole, fluconazole, itraconazole, and miconazole – on 14 *Malassezia pachydermatis* isolates obtained from dogs and 1 reference strain. A slightly modified M27–A3 method (CLSI 2008) and the checkerboard test (Nikolić et al. 2017) were used for this purpose. Our results show an additive effect of all 4 antifungals with manuka honey concurrent use. Based on the determined values of fractional inhibitory concentration index (FICI – 0.74±0.03 when manuka honey combined with clotrimazole, 0.96±0.08 with fluconazole, 1.0±0 with miconazole and 1.16±0.26 with itraconazole), it was found in all cases that the effect of substances used is more pronounced in mutual combination than when used separately.

Keywords: *Malassezia pachydermatis*, manuka honey, azole antifungals, synergy

Introduction

Malassezia pachydermatis is a commensal and occasional opportunistic yeast that is isolated from the skin of wild and domestic carnivores. It is a lipophilic yeast that colonizes the skin and mucous membranes. *M. pachydermatis* is a common secondary factor of otitis externa and can also lead to secondary *Malassezia* dermatitis for example in atopy. Such a diseases are common in dogs and less frequent in other animals (Angileri et al. 2019, Schlemmer et al. 2019, Cabanes 2021). The transition from commensal to pathogen is frequent in dogs in particular, and in cats to a lesser extent, so, cases of *Malassezia* otitis externa and dermatitis are commonly presented to veterinarians in small animal practice (Guillot and Bond 2020).

Malassezia dermatitis and otitis with secondary *Malassezia* infection usually require treatment with azole antimycotics for prolonged periods mostly combined with antibiotics (in case of intercurrent bacterial infection) and anti-inflammatory drugs (Bond 2010, Velegraki et al. 2015). More studies report the occur-

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Table 1. Data on dogs and ear-samples used.

No.	Gender	Breed	Age	Note
1	♀	Yorkshire Terrier	6 years	erythemato-ceruminous otitis
2	♂	Hungarian Vizsla cross	4 years	erythemato-ceruminous otitis
3	♂	Bichon Frise	8 years	erythemato-ceruminous otitis, changes on the skin
4	♀	Labrador Retriever	3 years	erythemato-ceruminous otitis
5	♂	Cross breed	4 years	erythemato-ceruminous otitis
6	♀	Border Terrier	6 years	erythemato-ceruminous otitis
7	♂	Cross breed	11 years	erythemato-ceruminous otitis
8	♂	Cocker Spaniel	4 years	erythemato-ceruminous otitis
9	♂	Dachshund	1 years	erythemato-ceruminous otitis
10	♀	Pug	3 years	erythemato-ceruminous otitis
11	♀	Pug	3 years	erythemato-ceruminous otitis
12	♂	Cavalier King Charles Spaniel	7 years	erythemato-ceruminous otitis
13	♂	Newfoundland dog	11 years	erythemato-ceruminous otitis
14	♂	Yorkshire Terrier	3 months	erythemato-ceruminous otitis

rence of strains that are resistant or less susceptible to azole antifungals (Bond 2010, Angileri et al. 2019), therefore it is appropriate to look for other therapy options. In the last decades, a popular possibilities are natural products – plant extracts, essential oils or their components, which are often part of medicinal preparations with an antifungal effect. Another attractive option is the use of honey, especially the manuka honey.

Manuka honey (MH) has been shown to have numerous therapeutic properties. It is produced by the European honeybee *Apis mellifera* which forages on the manuka tree (*Leptospermum scoparium*) that grows throughout New Zealand and south-eastern Australia (Masad et al. 2022). Manuka honey is a well-established antimicrobial, with a broad spectrum of activity that is attributed to the presence of methylglyoxal (Nolan et al. 2019). The presence of organic acids, phenolic acid, enzymes and bioactive peptides in honey contributes to a pronounced capacity of honey to cure and treat diseases of various etiologies. Manuka honey, compared to other honeys, shows a higher number of polyphenolic compounds and even higher antioxidant capacity (Bazaid et al. 2022). The established antimicrobial potential of MH has resulted in the development of various medical grade honeys and has been further explored for its synergistic activity alongside antibiotics. Manuka honey is known for its wound-healing, anti-microbial, anti-oxidant, anti-tumor and anti-diabetic properties (Bazaid et al. 2022, Nolan et al. 2022a).

The aim of our work was to estimate the antifungal efficacy of manuka honey in combination with conventional azole antifungals (clotrimazole, fluconazole, itraconazole, and miconazole) on *Malassezia pachydermatis* yeast isolates obtained from dogs with confirmed mycosis.

Materials and Methods

Isolates used

The study was performed on 14 clinical isolates of *M. pachydermatis* and one reference strain – *M. pachydermatis* CBS 1879 (CBS Utrecht, Holland). Samples of isolates were collected at various veterinary clinics of eastern Slovakia from 14 dogs of different breeds, genders and ages ranging from 3 months to 11 years (Table 1). All the samples were taken as a swab of external auditory canal from dogs with confirmed yeast otitis.

The species affiliation was verified on the basis of phenotypic (macroscopic and microscopic) and genotypic characteristics (PCR-RFLP).

Preparation of the inoculum

Each sample was inoculated onto a Sabouraud dextrose agar with chloramphenicol – SDA (HiMedia, Laboratories Pvt., Ltd., Mumbai, India) on a Petri dish at 35°C for 72 hours. As a control, the reference strain of *M. pachydermatis* CBS 1879 was used and processed in the same manner as the isolates.

After that time, about 2–3 colonies of *M. pachydermatis* isolates and reference strain were dispersed in a sterile physiological solution containing 0.1% Tween 80 to form suspension containing 10^6 CFU/mL,

which corresponds to 1.0 McFarland's unit. This suspension was subsequently diluted with Sabouraud dextrose broth containing Tweens 40 and 80 – SBT (HiMedia, Laboratories Pvt., Ltd., Mumbai, India) until the final concentration of 10^4 CFU/mL was reached, and further used in the following experiment.

Preparation of substances tested

Manuka honey has been procured as a mass-produced preparation of brand name Activon tube (Advancis Medical, United Kingdom). It contains 25 g of pure manuka honey of medicinal quality from New Zealand. According to the manufacturer's recommendations, it is intended for external use, primarily for the treatment of oozing and difficult-to-heal infected wounds and necrotic tissue. The product is originally filtered and sterilised, making it suitable for application to wounds.

Azole antimycotics, namely clotrimazole, fluconazole, itraconazole and miconazole were purchased as pure substances for laboratory use (Sigma Aldrich, Schnelldorf, Germany).

A 96-well, U-shaped bottom microtiter plates (Merck KGaA, Darmstadt, Germany) were required to prepare the microdilution broth assay. Two-fold dilutions of selected antifungals in SBT (50 µL in each well) were performed on them, reaching the concentrations from 64 – 1 µg/mL (columns 1 – 7). Column 8 was free of antifungal, it served only for MH solution and column 9 was a negative control, containing no isolate, whereas column 10 was a positive control, containing no tested compound. The MH was diluted in test tubes containing SBT to concentrations from 800 – 12.5 mg/mL. A concentration gradient of MH was then added into wells (rows A – G) to the antifungals situated in columns of the plate, diluting these solutions in half and the microplates thus prepared were ready to add the inoculum. Row H was free of MH, that is, it contained just the concentration gradient of antifungals.

MICs determination of tested compounds

The susceptibility of *M. pachydermatis* yeast cells to tested substances was determined by a partially modified M27–A3 method (CLSI, 2008) and the mutual effect of them (synergistic, additive, indifferent and antagonistic) was evaluated using the checkerboard test (Nikolić et al. 2017).

Microtiter plates, the preparation of which is mentioned in the previous section, were subsequently used for this purpose. To each well ranging from columns 1 – 10 (except for 9) and from rows A – H, 100 µL of inoculum were added, which led to second dilution of microplates content. So the final concentration of the tested substances corresponded to the following values: 16 – 0.25 µg/mL for each azole antimycotic and 200 – 3 mg/mL for MH, respectively. One microplate served for 1 isolate and one combination of antifungal agent with MH, so 64 plates were needed (4 combinations, 14 isolates and 1 reference strain tested in duplicate). Samples were incubated for 72 hours at 35°C and then the minimal inhibitory concentrations (MIC) were evaluated. For better visualization, 0.15% solution (10 µL) of resazurin dye was added for 12 hours. A checkerboard method (Nikolić et al. 2017) with slight modifications was used to evaluate the mutual effect of MH combined with azole antifungals. The mutual effects were evaluated on the base of fractional inhibitory concentration index (FICI). The FICI is defined by relation: $FICI = \frac{FIC1}{MIC1} + \frac{FIC2}{MIC2}$, where MIC1 is the MIC of antimycotic alone, FIC1 is the MIC of antimycotic combined, MIC2 is the MIC of the MH alone, FIC2 is the MIC of the MH combined. The results were interpreted as follows: FICI≤0.5 indicates the synergistic effect, FICI>0.5<2 indicates the additive effect, FICI≥2<4 indicates the indifferent effect and FICI>4 indicates the antagonistic effect.

Statistical analysis

The results of the MICs, FICs and FICIs were evaluated by the MS Excel statistical functions – minimum and maximum value (min – max), average (x), standard deviation (SD), median (Me) and mode (Mo). The statistical significance was assessed by the statistical program GraphPad Prism 5.0 (GraphPad software Inc. CA, USA) by using an unpaired t-test.

Results

In this study, the effectiveness of 4 azoles combined with manuka honey was tested on 14 isolates and 1 reference strain of *M. pachydermatis*. Table 2 shows the results of MIC values of clotrimazole combined with MH and their statistical analysis. It is obvious, that FIC of both, clotrimazole (2.14 µg/mL) and MH (82.14 mg/mL) was 2 – 4-times lower compared to their MIC ($p<0.0001$), that is, their interaction caused a supportive effect. This is also proven by the FICI value (0.62 – 0.75), which, based on the interpretation criteria mentioned above, corresponds to an additive effect.

Similar findings are also documented in Table 3, where, as can be seen, the combined effect of fluconazole with MH showed an encouragement. In this instance, although the mutual effect is additive, the FICI value (0.62 – 1) is slightly higher, which indicates

Table 2. Minimal and fractional inhibitory concentrations of clotrimazole combined with manuka honey.

Substance	Clotrimazole (µg/mL)		Manuka honey (mg/mL)		
Parameter	Alone (MIC1)	Combined (FIC1)	Alone (MIC2)	Combined (FIC2)	FICI
Isolates of *M. pachydermatis*, n=14					
min. – max.	8 – 16	1 – 4	100 – 200	50 – 100	0.62 – 0.75
x	9.14	2.14*	164.28	82.14*	0.74
SD	3.83	0.83	47.91	23.95	0.03
Mo	8	2	200	100	0.75
Me	8	2	200	100	0.75
M. pachydermatis CBS 1879, n=2					
min. – max.	8	2	200	100	0.75
x	8	2*	200	100*	0.75
SD	0	0	0	0	0
Mo	8	2	200	100	0.75
Me	8	2	200	100	0.75

Abbreviations: x – average, SD – standard deviation, Mo – mode, Me – median, FICI – fractional inhibitory concentration index, * – $p<0.0001$

Table 3. Minimal and fractional inhibitory concentrations of fluconazole combined with manuka honey.

Substance	Fluconazole (µg/mL)		Manuka honey (mg/mL)		
Parameter	Alone (MIC1)	Combined (FIC1)	Alone (MIC2)	Combined (FIC2)	FICI
Isolates of *M. pachydermatis*, n=14					
min. – max.	4 – 8	1 – 4	100 – 200	50 – 100	0.62 – 1
x	6	2.78*	185.71	92.85*	0.96
SD	2	1.08	34.99	17.49	0.08
Mo	8	2	200	100	1
Me	6	2	200	100	1
M. pachydermatis CBS 1879, n=2					
min. – max.	8	4	200	100	1
x	8	4*	200	100*	1
SD	0	0	0	0	0
Mo	8	2	200	100	1
Me	8	2	200	100	1

Abbreviations: x – average, SD – standard deviation, Mo – mode, Me – median, FICI – fractional inhibitory concentration index, * $p<0.0001$

Table 4. Minimal and fractional inhibitory concentrations of itraconazole combined with manuka honey.

Substance	Itraconazole (µg/mL)		Manuka honey (mg/mL)		
Parameter	Alone (MIC1)	Combined (FIC1)	Alone (MIC2)	Combined (FIC2)	FICI
Isolates of *M. pachydermatis*, n=14					
min. – max.	1 – 2	0.5 – 1	100 – 200	50 – 100	0.75 – 1
x	1.57	0.89*	178.57	100*	1.16
SD	0.49	0.38	41.03	32.73	0.26
Mo	2	1	200	100	1
Me	2	1	200	100	1
M. pachydermatis CBS 1879, n=2					
min. – max.	2	1	200	100	1
x	2	1*	200	100*	1
SD	0	0	0	0	0
Mo	2	1	200	100	1
Me	2	1	200	100	1

Abbreviations: x – average, SD – standard deviation, Mo – mode, Me – median, FICI – fractional inhibitory concentration index, * $p<0.0001$

Table 5. Minimal and fractional inhibitory concentrations of miconazole combined with manuka honey.

Substance	Miconazole (μg/mL)		Manuka honey (mg/mL)		
Parameter	Alone (MIC1)	Combined (FIC1)	Alone (MIC2)	Combined (FIC2)	FICI
Isolates of *M. pachydermatis*, n = 14					
min. – max.	1 – 2	0.5 – 1	100 – 200	50 – 100	1
x	1.5	0.75*	150	75*	1
SD	0.5	0.25	50	50	0
Mo	1	0.5	200	100	1
Me	1.5	0.75	150	75	1
M. pachydermatis CBS 1879, n = 2					
min. – max.	1	0.5	100	50	1
x	1	0.5*	100	50*	1
SD	0	0	0	0	0
Mo	1	0.5	100	50	1
Me	1	0.5	100	50	1

Abbreviations: x – average, SD – standard deviation, Mo – mode, Me – median, FICI – fractional inhibitory concentration index, * – $p<0.0001$

a slight decrease in the efficiency of combination used. The average value of the fluconazole´s minimum inhibitory concentration decreased from 6 μg/mL to 2.78 μg/mL and the amount of MH required to inhibit the growth of *M. pachydermatis* decreased from 185.71 mg/mL to 92.85 mg/mL ($p<0.0001$).

As shown in the Table 4, the mutual effect of itraconazole with MH is once again very similar to both clotrimazole and fluconazole combined with MH. The FICI values (0.75 – 1) are indistinctly higher than the previous one, which still points to an additive effect. Still though the average inhibitory concentration both of itraconazole and MH are higher when substances were used separately (1.57 μg/mL and 178.57 mg/mL) than when these were used in combination (0.89 μg/mL and 100 mg/mL, $p<0.0001$).

Inconspicuous differences occurred with miconazole, which seemed better when combined with MH. It's average values of MIC were lowered from 1.5 μg/mL to 0.75 μg/mL. The same applies to MH, whose MIC dropped by about half (150 mg/mL to 75 mg/mL, $p<0.0001$). Based on the FICI values (1), the mutual effect of tested substances was additive (Table 5).

Discussion

Manuka honey attracts the attention of scientists for its biological properties. These are the result of the presence of significant components. The major flavonoids in manuka honey are pinobanksin, pinocembrin and chrysin, while luteolin, quercetin, 8-methoxykaempferol, isorhamnetin, kaempferol and galangin have been also identified in minor concentration. Other constituents of interest are different 1, 2-dicarbonyl compounds, such as glyoxal (GO), 3-deoxyglucosulose (3-DG) and methylglyoxal (MGO) (Alvarez-Suarez et al. 2014).

Many significant effects of MH are documented in the scientific papers, starting with antioxidant, antibacterial, antiviral and ending with immunomodulating, anticancer or antidiabetic properties. It has been published that the marked antibacterial activity of MH directly originates from the contained MGO (Mavric et al. 2008).

The typical bacterial species which have been found to be sensitive to MH are Gram-positive (*Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus mutans, Streptococcus sobrinus, Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus, Staphylococcus, Enterococcus* spp., *Actinomyces viscosus*) and Gram-negative bacteria (*Stenotrophomonas maltophilia, Acinetobacter baumannii, Salmonella typhi, Pseudomonas aeruginosa, Proteus mirabilis, Shigella flexneri, Shigella sonnei, Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Burkholderia cepacia, Helicobacter pylori, Porphyromonas gingivalis, Campylobacter* spp.) (Alvarez-Suarez 2014). The sensitivity of several micromycetes (e.g. *Lichtheimia* sp., *Aspergillus flavus, Aspergillus fumigatus, Aspergillus terreus, Mucor circinelloides, Fusarium oxysporum, Exophiala* sp., *Apophysomyces* sp., *Actinomucor elegans*) was also proven (Yabes et al. 2017).

Interesting findings concern the use of MH in combination with antibiotics. The potentiating effect of these substances by MH was found. The improved growth inhibition and bactericidal activity was observed for 4 various manuka honeys (differentiated on the basis of MGO content) in combination with macrolide antibiotic azithromycin against *Mycobacterium abscessus* strains. The growth was inhibited by 0.037 g/mL manuka honey when used alongside azithromycin,

compared to 0.476 g/mL when used alone. MIC of azithromycin used alone was 4 µg/mL, however, MIC of azithromycin combined with MH was 1 µg/mL (Nolan et al. 2022a).

Moreover, MH works synergistically with amikacin, where it was shown that the addition of MH can lower the amikacin dosage whilst increasing its efficacy against *Mycobacterium abscessus* complex (Nolan et al. 2022b).

The synergy between MH and oxacillin has been ascertained by Jenkins and Cooper (2012). Growth of methicillin-resistant *S. aureus* (MRSA) was prevented by 0.25 mg/L oxacillin and 5% (w/v) MH in comparison to applying them separately (64 mg/L and 6%, respectively).

Additional results show that the MH–rifampicin combination is more effective than using oxacillin, fusidic acid, clindamycin, and gentamicin against *S. aureus* biofilms. MH and rifampicin were strongly synergistic in their ability to reduce both biofilm biomass and the viability of *S. aureus* cells at a level that is likely to be significant *in vivo*. Other combinations showed a minor synergistic activity (MH–fusidic acid) and antagonistic effects when MH used in sub-inhibitory concentration, but not antagonistic when MH used at the inhibitory concentration (MH–clindamycin, MH–gentamicin and MH–oxacillin) (Liu et al. 2018).

Several papers have already dealt with the effect of MH on *Candida* yeast. Although there are too few of them, nevertheless, Fernandes et al. (2020) found effectiveness of MH on both planktonic and biofilm cells of *Candida* species (*C. albicans, C. glabrata, C. parapsilosis* and *C. tropicalis*). They verified that MH at 50% (w/v) induced inhibition of a range of pathogenic *Candida* species in planktonic state. Comparing the minimal inhibitory and minimal fungicidal concentration, *C. tropicalis* was the most susceptible to MH. Concerning 24 h biofilms assays, for *C. albicans*, a significant reduction of the biofilm occurred as compared with control, after treatment with MH at 50%, however the effect on 48 h biofilms was obtained only at 75%.

In a single study, the minimal fungicidal concentrations of various topical preparations, including MH, were determined, among others, on *M. pachydermatis*. After all, the values were not detectable for MH at any dilution at either time point (Uri et al. 2016).

Unfortunately, no article reports synergism studies with other compounds or antifungals on yeasts. In our study, we noted an additive effect of MH with all 4 antifungals – clotrimazole, fluconazole, itraconazole and miconazole, respectively. *M. pachydermatis* planktonic cells, whether isolates or a reference strain, exposed to MH and the tested antifungals were more sensitive to their combined effect than to the effect of each agent applied separately. Some of the antifungals tested by us are also used topically in veterinary medicine (miconazole, clotrimazole) and their use with MH seems theoretically advantageous. However, the practical implementation is questionable, as the application of honey to the skin (*Malassezia* dermatitis and otitis) would be inconvenient, moreover, the sweet taste of honey would tempt dogs to lick the affected skin areas. Another disadvantage could be the gluish consistency of honey, which would stick the dog hair, and ultimately, it´s scent would attract the insects. For this reason, it would be useful to research the antifungal effect of the components (especially MGO) present in manuka honey.

Conclusion

From the presented work and the results obtained from our research, it can be seen that manuka honey, which is known to have proven antibacterial effects, is also effective against *Malassezia pachydermatis* yeast, at an average concentration of 150 mg/mL. Moreover, when used in combination with conventional azole antifungals used in practice – clotrimazole, fluconazole, itraconazole and miconazole, it shows an additive effect.

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