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Antimicrobial effects of propolis on *Clostridium difficile* belonging to the different PCR-ribotypes

SUMMARY

Clostridium difficile is a Gram-positive, anaerobic bacilli which is a primary cause of nosocomial diarrhoea. The rising incidence of antibiotic resistance in pathogens such as *C. difficile* makes research for new treatments important and necessary. It has been observed that propolis has bactericidal effects. Propolis is a natural resinous hive product that is manufactured by honeybees (*Apis mellifera*). The antimicrobial activity of propolis is an important biological property. Twenty strains of the *C. difficile* belonging to four prominent PCR-ribotypes (RT) (RT017, RT023, RT027, and RT046) were used in research. MBC value were determined by broth dilution method. Propolis samples were obtained from honey bees farm in Lublin voivodship. Bactericidal effect of propolis on *C. difficile* strains was observed at 3.9 mg/ml concentration.

Keywords: propolis, minimal bactericidal concentration (MBC), *Clostridium difficile*

STRESZCZENIE

Clostridium difficile gram dodatnia beztlenowa laseczka powodująca zakażenia szpitalne, jest zaliczana współcześnie do grupy najbardziej opornych na antybiotyki drobnoustrojów. To zjawisko ogranicza możliwości terapeutyczne i wymusza potrzebę poszukiwania alternatywnych, naturalnych środków o działaniu przeciwbakteryjnym. Naturalnym środkiem biologicznie czynnym, wykazującym działanie przeciwdrobnoustrojowe jest produkt pszczeli propolis,

substancja żywiczna wywarzana przez pszczoły miodne (*Apis mellifera*). Badaniem objęto 20 klinicznych szczepów *C. difficile* należących do 4 różnych PCR-rybotypów występujących w Polsce (017, 023, 027, 046). Wartości MBC określono, wykorzystując metodę seryjnych rozcieńczeń w podłożu płynnym. Próbki propolisu pochodziły z gospodarstwa pszczelarskiego położonego w województwie lubelskim. Propolis wykazał działanie bakteriobójcze wobec badanych szczepów *C. difficile* przy stężeniu 3,9 mg/mL.

Słowa kluczowe: propolis, minimalne stężenie bakteriobójcze, *Clostridium difficile*

INTRODUCTION

Clostridium difficile is a Gram-positive, strictly anaerobic, spore forming bacteria and the major cause of nosocomial diarrhea. This microorganism is an etiological agent for several clinical complications, including pseudomembranous colitis, toxic megacolon and intestinal perforation, which all have relatively high death rates. The major risk factor for developing CDI after contamination is the imbalance of the microbiota, which leads to the disruption of its barrier effect. This occurs mainly after use of broad-spectrum antibiotics. Infection affects primarily elderly, patients undergoing surgery, long-term hospitalized patients and immuno-compromised patients (14). Main virulence factors of *C. difficile* are the high molecular weight toxins: A (TcdA); 308kDa and B (TcdB); 270 kDa, which are essential for the disease manifestations. In addition, some strains produce a binary toxin (CDT) expressed by some strains, including epidemic lineages which is not clearly established, although it has been related to higher morbidity and mortality (8). Other factors such as fibronectin binding protein A, surface layer proteins (SPLs), cell-wall proteins (CWPs), flagella and cysteine protease Cwp84 have been involved in *C. difficile* colonization (19). Asymptomatic carriage of toxigenic strains on hospital admission is frequent, around 8% as estimated by a recent meta-analysis, and the risk of developing a *C. difficile* infection (CDI) for colonized patients is significantly higher compared with non-colonized patients. Infants are also commonly asymptomatically colonized with pathogenic strains and could represent a potential reservoir for bacteria transmission (4). In Europe, the number of CDI cases is increasing due to hyper-virulent strains, mainly belonging to the polymerase chain reaction (PCR) ribotype (RT) 027 (4). In a survey of CDI cases in Poland (2011–2013), *C. difficile* PCR RT027 was found to be the most prevalent PCR RT in all the participating hospitals. This strain is characterized by higher *in vitro* production of toxin A and B and presence of the binary toxin gene (13).

The rising incidence of antibiotic resistance of nosocomial pathogens such as *C. difficile* makes research for new treatments important and necessary. Propolis is a natural resinous hive product that is manufactured by honeybees (*Apis mellifera*) from natural balsamic resins. It is actively secreted by plants on leaf buds and barks, in combination with bee wax and bee secretions (18). Propolis is prepared by the honeybees to seal the cracks, smooth walls and to keep moisture and temperature. Because of antimicrobial activity and anti-inflammatory properties of propolis, inhabitants in bee hives are protected from bacterial, fungal and viral infections. Raw propolis is typically composed of 50% plant resins, 30% bee waxes, 10% essential and aromatic oils, 5% pollens and 5% other organic substances including vitamins such as vitamin A, B1, B2, B6, C, D, E, nicotinic acid and folic acid as well as macro and trace minerals such as calcium, magnesium, iron, copper, zinc, manganese, nickel, cobalt, vanadium and strontium (1) (Fig. 1).

Depending on the geographic origin, propolis may contain 250–350 components. Major group identified in the propolis are flavonoides, phenolic acids and their esters and terpens. Propolis from Europe, China and Argentina is highly abundant in chrysin (2–4%), pinocembrin (2–4%), pinobanksin-acetate (1.6–3%) and galangin (1–2%). The total flavonoid content of propolis may be used as

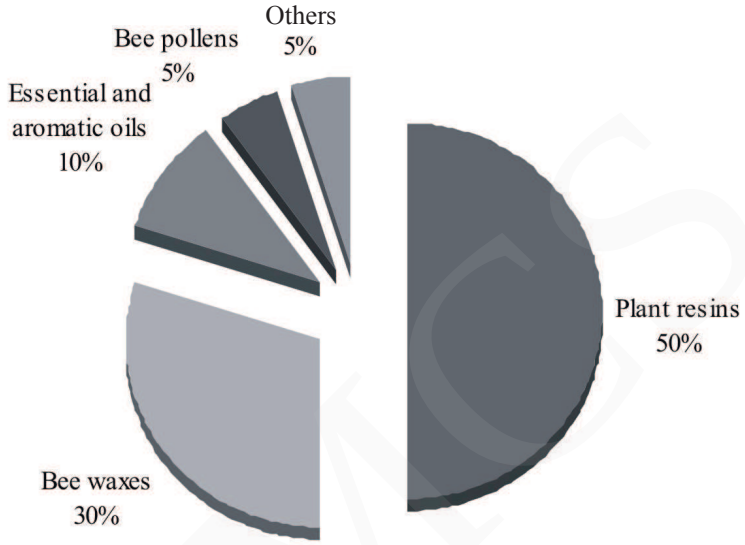

Fig. 1. Composition of raw propolis, after (1).

a quality index, classifying 11–14% as acceptable, 14–17% as good, and more than 17% as high quality. It has been reported that propolis is collected from resins of poplars, conifers, birch, pine, alder, willow, palm, *Baccharis dracunculifolia*, and *Dalbergia ecastaphyllum.* Propolis is widely used to prevent and treat colds, wounds and ulcers, rheumatism, sprains, heart disease, diabetes and dental caries due to its diverse biological properties such as anti-inflammatory (7). Biologically active ingredients of the propolis are presented in Table 1.

Table 1. Biologically active ingredients in propolis, after (2, 5)

Biological activity	**Active substance**
Antibacterial	Flavonones, flavons, phenolic acids and their esters
Antifungal	Pinocembrin, galangin, benzoic acid, salycilic acid, vanillin
Antiviral	Polyphenols, phenyl-carboxylic acids and esters, cinnamic acids, caffeic acid, quercetin, luteolin, fisetin, quertecagetin
Antioxidant	Flavonoids phenolics and their esters
Hepatoprotective	Flavonoids, caffeic acid phenethyl ester (CAPE), ferulic acid, caffeic acid
Anticancer and antitumor	CAPE, caffeic acid, apigenin, quercitin, genistein, rutin, p-coumaric acid, ferulic acid, kampferol
Immuno modulating	CAPE, chrysin, benzylcaffeate, phenethylferrulate, cinamic acid
Anti-inflammatory	Flavonones, flavons, phenolic acids and their esters
Anti-ulcer	CAPE, caffeic acid, pinocembrin, galangin, chrysin

The antimicrobial activity of the propolis is the most important biological property. The antibacterial activity has been demonstrated against both gram-positive and gram-negative bacteria such as *Staphylococcus aureus* (11), *Enterococcus* spp. (11), *Pseudomonas aeruginosa* (11), *Streptococcus* spp. (10), *Escherichia coli* (17), *Helicobacter pylori* (3). In spite of the large composition differences of the propolis type, all have antimicrobial activity. It seems that all of the propolis components are responsible for the bactericidal action rather than individual substances (2, 5).

The aim of this study is to explore effects of propolis produced in Poland on the *C. difficile* strains belonging to the different ribotypes.

MATERIALS AND METHODS

BACTERIAL STRAINS

Twenty *C. difficile* toxigenic strains belonging to four different PCR RTs, including RT017 (A-B+CDT-), RT023 (A+B+CDT+), RT027 (A+B+CDT+) and RT046 (A+B+CDT-) randomly selected, were used in the study. The strains were collected from the Anaerobic Laboratory (AL) in the Department of Medical Microbiology, Medical University of Warsaw, and were frozen at −70°C in a Microbank ™ bacterial storage system (Pro-Lab Diagnostics, UK). The strains were thawed before the experiments, cultured on Columbia Agar plates (bioMérieux, France) and incubated at 37 °C for 48 h under anaerobic conditions. Isolates were confirmed to be *C. difficile* as described previously (13). PCR RTs were determined using methods as described by Stubbs et al. (15).

PREPARATION OF DIMETHYLSULFOXIDE EXTRACT OF PROPOLIS DEP

Propolis samples were obtained from honey bees farm in Lublin voivodship. Samples were ground and 5 g of the ground propolis was dissolved in 5 mL of dimethyl sulfoxide (100% w/v) (DMSO) (Sigma, USA ≥ 99.5%) by continuous mixing for 5 h. It was then incubated at 37°C overnight and centrifuged at 800 × g for 15 min. To remove waxes and less soluble substances, the suspensions were subsequently frozen at –20°C for 24 h, then filtered with filter paper and 0.2 µm filter (Nunc, Denmark), and 1000 mg/mL extract was obtained. The working stocks (125 mg/mL, 62.5 mg/mL, 31.25 mg/mL, 15.625 mg/mL, 7.812 mg/mL, 3.9 mg/mL 1.95 mg/mL) were prepared using brain-heart infusion medium (BHI; Difco, USA).

DETERMINATION OF THE MBC OF PROPOLIS FOR THE DIFFERENT *C. DIFFICILE* STRAINS

The working stocks of propolis (125 mg/mL, 62.5 mg/mL, 31.25 mg/mL, 15.625 mg/mL, 7.812 mg/mL, 3.9 mg/mL 1.95 mg/mL) were inoculated with 100 µL of the overnight *C. difficile* strains and incubated at 37 °C for 48 h under anaerobic conditions. After 48 h, the turbidity of the tubes was analyzed, which is an indicator of bacterial growth. The 20 µL of the lowest concentration of propolis that showed no turbidity was pipetting into fresh Columbia Agar plate with 5% sheep blood (Oxoid, UK), which were incubated at 37°C for 48 h under anaerobic conditions. The lowest concentration with no detectable bacterial growth was defined as the MBC. If growth was detected at the highest concentration of propolis used, then a higher concentration was checked. Positive control was inoculated BHI, negative control was fresh BHI medium (12). Experiments were repeated two times for each PCR RT strain (RT017, RT023, RT027 and RT046) and the duplicate values were averaged.

RESULTS

The effectiveness of propolis against medically relevant microorganisms suggests that it could potentially be used as an alternative therapy. Studies have been conducted to examine the antimicrobial effect of propolis against many bacteria, excluding *C. difficile*. Minimal bactericidal concentration of propolis on the tested *C. difficile* strains were found to be between 3.9–31.2 mg/mL (Tab. 2).

Tab. 2. MBC values of propolis against the examined *C. difficile* strains

No.	PCR-ribotype	MBC propolis (mg/mL)
1	017	7.8
2	017	3.9
3	017	3.9
4	017	3.9
5	017	3.9
6	023	3.9
7	023	15.6
8	023	3.9
9	023	3.9
10	023	3.9
11	027	31.2
12	027	3.9
13	027	3.9
14	027	3.9
15	027	3.9
16	046	7.8
17	046	3.9
18	046	3.9
19	046	3.9
20	046	3.9

One of the examined *C. difficile* strains had higher MBC than others (31.2 mg/mL) and belonged to the PCR-RT 027. It may be associated with the hyper-virulent status of these strains. The lowest MBC characterized *C. difficile* strains belonging to the PCR RT 017 and 046. Starzyk and Doleżal (16) reported susceptibility to the Polish propolis of clinically important strains, isolated from hospitalized patients. The mean MBC of propolis on *S. aureus* was found to be 3–4 mg/mL, *E. coli* 6 mg/mL, *K. pneumoniae* 5–6 mg/mL, *P. vulgaris* 5–6 mg/mL, *P. aeruginosa* 6–7 mg/mL. These results indicate the slightly higher sensitivity to propolis of Gram-positive than Gram-negative bacteria. Wojtyczka et al. (9) tested the difference between susceptibility of MSSA and MRSA to propolis. The MBC

average values were similar for MRSA and MSSA strains, and found to be 2.35 ± 1.21 mg/mL and 2.35 ± 0.86 mg/mL, respectively.

The results of the presented studies show that the microbial activity of propolis on different bacteria is similar. This demonstrates that bees collect propolis from such plants which provide strong antimicrobial activity of propolis, necessary for hygiene of the hive to protect it from the development of microorganism harmful to the bees community. In conclusion, we demonstrated that propolis has bactericidal effect on clinically significant strains of *C. difficile*, including those belonging to PCR RT027. It could potentially be used as an alternative therapy. Especially when this year for the first time was performed an alternative therapeutic intervention with manuka honey on the therapy resistant *C. difficile* infection (6). This case report demonstrates the possible therapeutic value of honey and its derivatives as a treatment for therapy-resistant CDI.

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