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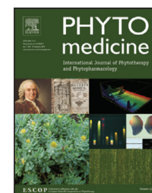

Original Article

Are Russian propolis ethanol extracts the future for the prevention of medical and biomedical implant contaminations?

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ABSTRACT

Background: Most studies reveal that the mechanism of action of propolis against bacteria is functional rather than structural and is attributed to a synergism between the compounds in the extracts.
Hypothesis/Purpose: Propolis is said to inhibit bacterial adherence, division, inhibition of water-insoluble glucan formation, and protein synthesis. However, it has been shown that the mechanism of action of Russian propolis ethanol extracts is structural rather than functional and may be attributed to the metals found in propolis. If the metals found in propolis are removed, cell lysis still occurs and these modified extracts may be used in the prevention of medical and biomedical implant contaminations.
Study design: The antibacterial activity of metal-free Russian propolis ethanol extracts (MFRPEE) on two biofilm forming bacteria: penicillin-resistant *Staphylococcus aureus* and *Escherichia coli* was evaluated using MTT and a Live/Dead staining technique. Toxicity studies were conducted on mouse osteoblast (MC-3T3) cells using the same viability assays.
Methods: In the MTT assay, biofilms were incubated with MTT at 37 °C for 30 min. After washing, the purple formazan formed inside the bacterial cells was dissolved by SDS and then measured using a microplate reader by setting the detecting and reference wavelengths at 570 nm and 630 nm, respectively. Live and dead distributions of cells were studied by confocal laser scanning microscopy.
Results: Complete biofilm inactivation was observed when biofilms were treated for 40 h with 2 µg/ml of MFRPEE. Results indicate that the metals present in propolis possess antibacterial activity, but do not have an essential role in the antibacterial mechanism of action. Additionally, the same concentration of metals found in propolis samples, were toxic to tissue cells. Comparable to samples with metals, metal free samples caused damage to the cell membrane structures of both bacterial species, resulting in cell lysis.
Conclusion: Results suggest that the structural mechanism of action of Russian propolis ethanol extracts stem predominate from the organic compounds. Further studies revealed drastically reduced toxicity to mammalian cells when metals were removed from Russian propolis ethanol extracts, suggesting a potential for medical and biomedical applications.

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Introduction

Propolis is a resinous substance processed by honeybees from the native vegetation near their hive (Teixeira et al., 2008). More specifically, it is a complex mixture of components collected by honeybees from buds or exudates of plants (resin), beeswax, pollen and sugars. Propolis exhibits a variety of biological activities including bactericidal, antiviral, fungicidal, anti-tumor, antioxidant, and anti-inflammatory properties (Park et al., 2000; Duran et al., 2006; Medic-Saric et al., 2009; Parolia et al., 2010). This naturally-

Abbreviations: MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ATCC, American Type Culture Collection; TSBG, Tryptic soy broth supplemented with 0.2% glucose; PBS, phosphate buffered saline; S. aureu, Staphylococcus aureus; E. coli, Escherichia coli; SEM, scanning electron microscopy; SDS, sodium dodecylsulfate; RPEE, Russian propolis ethanol extracts; MFRPEE, Metal-free Russian propolis ethanol extracts; DPPC, dipalmitoylphosphatidylcholine; DPPS, dipalmitoylphosphatidylserine; EtOH, ethanol; and LUVs, Large unilamellar vesicles; LC-MS, Liquid chromatographic mass spectrometry.

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produced antibacterial resin is currently a popular alternative medicine in various parts of the world, including Asia and Europe (Bankova et al., 2000). The composition of propolis is originated from three possible sources: Extracts collected by bees from the secretion of plants, the excretions from the metabolism of bees, and other materials, that are introduced during the preparation of propolis (Marcucci 1995; Daugsch et al., 2008).

Propolis contains more than 200 different compounds (Marcucci 1995; Sforcin et al., 2000; Banskota et al., 2001; Brooks et al., 2002; Silici and Kutluca 2005) that conprise substituted phenolic acids and esters, flavonoids, amino acids, aliphatic acids, aromatic esters and acids, fatty acids, carbohydrates, aldehydes, amino acids, ketones, chalcones, dihydrochalcones, terpenoids, vitamins (B1, B2, B6, C, and E), metals (aluminum, calcium, cesium, copper, iron, lithium, manganese, mercury, nickel, silver, vanadium, potassium, sodium, magnesium, and zinc), and beeswax (De Castro 2001; Volpi and Bergonzini 2006; Yoshimi et al., 2007; Yasuyuki et al., 2010).

Bacteria are highly adaptive organisms that have evolved a distinct ability to thrive, even in the most hostile environments (Cos et al., 2010). Within biofilms that act as a physical barrier, bacteria protected by the extracellular matrix can acquire plasmid-encoded multidrug resistance genes, which negate bactericidal properties to various antimicrobial agents (Costerton et al., 1999; Chapman 2003; Langsrud et al., 2003). The resistant nature of biofilms make them extremely challenging to treat, despite the host immune system response and antibiotics (Donlan and Costerton 2002). Once matured, these bacterial infections lead to increased physical suffering, prolonged hospital visits, implant rejection, device failure, recurrent operations, and even death (Murtough et al., 2002). There are a number of constituents in propolis and its extracts that are known to have biological activity and can be used to inactivate difficult-to-treat infections, therefore minimizing the spread of antibiotic resistance.

The anti-biofilm activity, along with a new mechanism of action for Russian Propolis Ethanol Extracts (RPEE) has already been proven against *S. aureus* biofilm and *E. coli* biofilms (Bryan et al., 2015). Even with these findings, the inactivation of bacterial biofilms by means of propolis is still relatively novel, with many unanswered questions, including the antibacterial effects of metals found in propolis samples. Although there are no definitive examples in the literature of propolis components binding to heavy metal ions for improving anti-biofilm activity, it is believed that the presence of metals in RPEE does contribute to the antibacterial activity, but to what extent has not been determined yet. The results discussed in this paper begin to explore: 1) the antibacterial effects of metals on biofilms; and 2) RPEE as an alternative treatment for future medical and biomedical applications.

Materials and methods

Bacterial strains and medium

Staphylococcus aureus (penicillin resistant, ATCC 29213), and *E. coli* (ATCC 25404), all good biofilm-forming strains, were purchased from the American Type Culture Collection (ATCC, Manassas, VA USA). Biofilms were grown in tryptic soy broth (TSB) supplemented with 0.2% glucose (TSBG).

Reagents and solutions

A Live/Dead staining kit was purchased from Invitrogen Life Technologies (Carlsbad, CA USA) for the staining of bacteria within biofilms. Also, 5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), in phosphate buffered saline (PBS), sodium dodecylsulfate (SDS), ethylenediaminetetraacetic acid (EDTA), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS), and other reagents were all purchased from the Sigma Chemical Laboratory (St Louis, MO USA).

Growth of biofilms on different materials

For each experiment, an isolated single bacterial colony was picked from an agar plate, transferred to 10–15 ml of TSBG medium and then incubated under orbital agitation (100–150 rpm) at 37 °C for 18–24 h The overnight culture of both *S. aureus* and *E. coli* was diluted in TSBG to 2×10^6 cells/ml and then inoculated on surfaces of different materials including polyethylene terephthalate, 8-well glass chambers, polystyrene 6-well plates, and silicon wafers. *S. aureus* biofilms 20–24 µm thick and *E. coli* 15–20 µm thick were formed on all tested materials within 18 h and were used throughout this study.

Preparation of RPEE

The Russian propolis was gathered from the Krasnodar Krai region of Russia, which is located in the southwestern part of the North Caucasus. Hand-collected propolis samples were kept desiccated and in the dark up until processing as previously described (Bryan et al., 2015).

Antibacterial activity studies of RPEE

It is known that planktonic bacteria once adhered to a surface can form biofilms. In biofilm inactivation assays, 5 ml of mid-logarithmic (mid-log) phase bacteria ($\sim 2 \times 10^6$ cells/ml) suspended in TSBG medium were used to grow biofilms. After 18 h of incubation, the formed biofilms were washed with PBS in order to remove planktonic and loosely attached bacteria. All RPEE were weighed under aseptic conditions in sterile volumetric flasks and 50 µl (10 µg) of RPEE were pipetted into 5 ml of fresh TSBG. These TBSG solutions containing the 2 µg/ml propolis ethanol extract were used to treat bacteria biofilms. After exposure to propolis extracts for 40 h, TSBG-containing propolis was removed, and the biofilms were once again washed with PBS. Throughout this study, all bacterial biofilm control groups (0 µg/ml or EtOH) were subjected to TSBG culture media containing 10 µl/ml of HPLC, gradient grade, ≥99.9% ethanol, which was used in the extraction of propolis. In these control groups, almost 98% of *S. aureus* planktonic bacteria and the *S. aureus* bacteria in biofilms were still alive.

In the MTT assay, biofilms were incubated with MTT at 37 °C for 30 min. After washing, the purple formazan formed inside the bacterial cells was dissolved by SDS and then measured using a microplate reader by setting the detecting and reference wavelengths at 570 nm and 630 nm, respectively (Kharidia and Liang 2011).

Staining of live and dead bacteria

In order to visually confirm the results obtained from the MTT method, another cell viability assay using a Live/Dead staining kit was used to evaluate the antibacterial activity of the different propolis samples. Live and dead bacterial distributions of bacteria in biofilms were studied by confocal laser scanning microscopy using a Live/Dead staining kit as described previously (Traba and Liang 2011). Biofilms grown on LabTek 8-well cover-glass chambers were washed with PBS to remove planktonic bacteria and TSBG medium. Next, Live/Dead dyes in PBS were added and incubated for 15 min at room temperature. The excitation wavelength was fixed at 488 nm, and the emission wavelengths were set at 505–530 nm (for the live cells) and >560 nm (for the dead cells).

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