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Extraction Method Plays Critical Role in Antibacterial Activity of Propolis-Loaded Hydrogels

Gabriel G. de Lima [1], Ronaldo O. de Souza [1], Aline D. Bozzi [1], Malgorzata A. Poplawska [1], Declan M. Devine [1, 2], Michael J.D. Nugent [1, *]

[1] *Materials Research Institute, Athlone Institute of Technology, Athlone, Ireland*
[2] *Rehabilitation Medicine Centre, Mayo Clinic, Rochester, Minnesota 55902*

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ABSTRACT

Extracted propolis has been used for a long time as a remedy. However, if the release rate of propolis is not controlled, the efficacy is reduced. To overcome this issue, extracted propolis was added to a cryogel system. Propolis collected from southern Brazil was extracted using different methods and loaded at different concentrations into polyvinyl alcohol (PVA) and polyacrylic acid hydrogels as carrier systems. The material properties were investigated with a focus on the propolis release profiles and the cryogel antibacterial properties against 4 different bacteria namely: *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas putida*. Swelling studies indicated that the swelling of the hydrogel was inversely related to propolis content. In addition, propolis release studies indicated a decreased release rate with increased propolis loading. PVA and PVA/polyacrylic acid–loaded propolis were effective against all 4 bacteria studied. These results indicate that the efficacy of propolis can be enhanced by incorporation into hydrogel carrier systems and that hydrogels with higher concentrations of propolis can be considered for use as bactericide dressing.

Introduction

Hydrogels are 3D polymer networks that can swell in aqueous solutions. Hydrogels have many characteristics that make them excellent drug delivery vehicles such as their mucoadhesive and bioadhesive properties that have been harnessed to enhance drug resistance time and complement tissue permeability.[1,2] In addition, the composition and properties of hydrogels can be tailored for specific applications.[3,4] Indeed, natural and biodegradable polymers have been extensively explored because of the ability to fabricate biomaterials with bespoke properties.[5,6] Furthermore, recently developed hydrogels have self-healing abilities that are triggered once the structure is damaged.[7,8] One hydrogel that is frequently used for pharmaceutical applications is polyvinyl alcohol (PVA) which is transparent, malleable, bioinert, and biocompatible.[9] PVA, as a synthetic polymer can be cross-linked by a variety of methods, such as chemical crosslinking,[10] irradiation[11] in addition to the freeze-thaw technique.[12] PVA hydrogels have a high resistance to solvents, oils, and greases; superior resistance to oxygen permeability compared to other polymers and are also an excellent adhesive.[13]

Freeze and/or thawed gels, or cryogels, are formed by freezing polymeric solutions. On freezing the solvent, crystals grow and impinge on adjacent growing crystals. On thawing, a porous system is created. The main advantages of PVA cryogels include biodegradability and biocompatibility because there is no solvent involved during processing.[14] Furthermore, by adding specific polymers such as those containing pendant acid or basic chemical moieties, pH-sensitive hydrogels can be created. These systems have the advantage that controlled release of protons can be achieved based on the response to changes in the pH of the environment. Furthermore, the addition of pH sensitive polymers, such as, polyacrylic acid (PAA) to PVA can be used to modulate drug release from hydrogels with bioadhesive properties, which is relevant when used in the preparation of transdermal patches for treatments of dermatological diseases.[15]

The natural substance propolis, collected by *Apis mellifera* bees and harvested from derived plants, has been used in medicine for centuries,[16] as it has a role in protection against the entry of microorganisms, fungi, and bacteria.[17-20] The composition of propolis is dependent of the flora, season, and time of the collection.[21] The

* *Correspondence to:* Michael J.D. Nugent (Telephone: +353 9064 68172; Fax: +353 0964 68148).
E-mail address: mnugent@ait.ie (M.J.D. Nugent).

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antibacterial activity of propolis is attributed to the inherent presence of phenols and flavonoids (flavones, flavanones, flavonols, dihydroflavonols, chalcones).[16] However, for biomedical applications, purification is necessary to remove inert components (wax, ash, bioactive compounds, and pollen) while simultaneously preserve active components (phenols and flavonoids). Several methods are reported, such as maceration (MAC), ultrasound-assisted extraction in addition to Soxhlet extraction (SOX).[22,23] Propolis extractions are commonly reported using 70%-80% ethanol.[24-26] However, ethanol extraction has disadvantages, such as the presence of a strong and unpleasant taste together with high ethanol residue.[18,27] These disadvantages result in difficulties in packaging, transport, and incorporation in other dosage form. Thus, extracted propolis alone is not suitable for medical and pharmaceutical applications. To overcome these problems, membranes and hydrogels incorporating propolis have been developed for wound care and have been shown to be effective as antimicrobial agents with superior bone repair properties.[28,29]

Few reports are published on the use of PVA cryogels loaded with propolis. Oliveira et al.,[30] investigated the antimicrobial activity on different types of bacteria using PVA cryogels containing a commercial propolis extract. In particular, this study examined the affect of hydrogel microstructure and mechanical properties on propolis release on swelling, where the results identified *S. aureus* as the only bacterial strain susceptible effective to the propolis extract.

To study the potential of PVA cryogels loaded with propolis in more detail, this work investigates the effects of various concentrations of different ethanol extractions on the mechanical, kinetic, and antimicrobial properties of PVA and pH-sensitive PVA/PAA cryogels.

Experimental

Materials

Polyvinyl alcohol, polyacrylic acid, and phosphate buffer solution (PBS) were supplied by Sigma-Aldrich, Ireland.

Propolis

Raw propolis was collected from *Apis mellifera* hives located in Quitandinha in the state of Paraná (PR), Brazil in Spring 2013 from *Baccharis uncinella* flora.

Methods of Phenolic Extraction From a Raw Propolis

Three methods including MAC, SOX, and SON were applied and compared to obtain a high-extraction efficiency of phenolic components from raw propolis. In each case, propolis was ground to a fine powder with 1 g (dry weight) dissolved in 70% ethanol at a ratio of 1:25 (w/v), as previously described in the literature.[31]

Maceration, Soxhlet, and Ultrasound-Assisted Extraction

MAC was performed at room temperature under constant stirring using a magnetic stirrer for 24 h.[26]

SOX was performed according to Cunha et al.[24] using a slightly altered method. Pulverized raw green propolis (4 g) was placed inside a paper thimble and subjected to SOX for 6 h at a maximum temperature of 65°C, using 100 mL of solvent.

SON was performed by placing a propolis solution in ethanol into an ultrasonic bath at 70°C for 1 h (Branson Ultrasonic Bath 2510).[26]

After the extractions, all solutions were filtered through a filter paper under vacuum.

SON and MAC extractions were stored overnight in a refrigerator to induce crystallization of dissolved waxes and then filtered 0°C to remove waxes from extract.[24]

At the end of the procedure, the extracts were stored in sterile amber glass flasks.

Chemical Analysis of Extracted Propolis

Ultraviolet-visible (UV-VIS) spectra of extracted propolis samples were recorded by diluting in a proportion of 1 mL of propolis/100 ml of ethanol. The mixture was scanned at 200-500 nm by UV-spectrophotometer (UV Jenway 7305).

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of each agent was determined using the agar dilution method as previously described.[32] MIC values were determined using Soxhlet propolis extract in a range of concentrations: 0.11, 0.25, 0.43, 0.67, 1.00, 1.22, 1.50, 1.86, 2.33, and 4 mg/mL. Control plates containing serial dilutions of ethanol alcohol were also tested using 8 technical replicates.

Polymeric Composition Formulation and Fabrication of Composites

Physically cross-linked (PVA) hydrogels loaded with propolis were prepared by dissolving known concentrations of PVA, with average molecular weight of 1,95,000 and a 98% hydrolysis concentration (w/v) in a total volume of distilled water together with range of concentrations of ethanol extracted propolis at 70°C with constant stirring until complete solubilization of the PVA was observed.

Another batch of samples was produced by adding PAA to the solubilized PVA solution (with a molecular weight of 3,000,000) at 50% concentration (w/w) at ambient temperature.

Finally, the samples were rapidly frozen to a constant temperature of −80°C for 2 h in an ultralow temperature freezer (Innova U535). The frozen solutions were then thawed in an oven to a temperature of 25°C with n = 10 technical replicates. Subsequently, samples were dried in an oven for 24 h at 30°C. The chemical reaction showing hydrogel synthesis is shown in Figure 1.

Microstructural Analysis

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-TFIR) was carried out on a Perkin Elmer Spectrum One fitted with a universal ATR sampling accessory. All data were recorded in the spectral range of 4000-650 cm^{-1} using 16 scan per sample cycle. Subsequent analysis was carried out using Spekwin32 software.

Kinetics of Hydrogels

Swelling studies of the propolis hydrogel composite samples were carried out using buffer solution at pH 7.4. To measure the swelling kinetics, preweighed samples were immersed in distilled water. Excess surface water was gently removed with paper, and the swollen samples were weighted at various time intervals over a 24-h period. The percentage swelling of a hydrogels was determined using Equation 1,

$$S(\%) = \frac{(W_s - W_d)}{W_d} \times 100, \quad (1)$$

where S (%) is the swelling ratio at any specific time, and W_d is the dried mass of the hydrogel before beginning the swelling studies.[33]