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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]

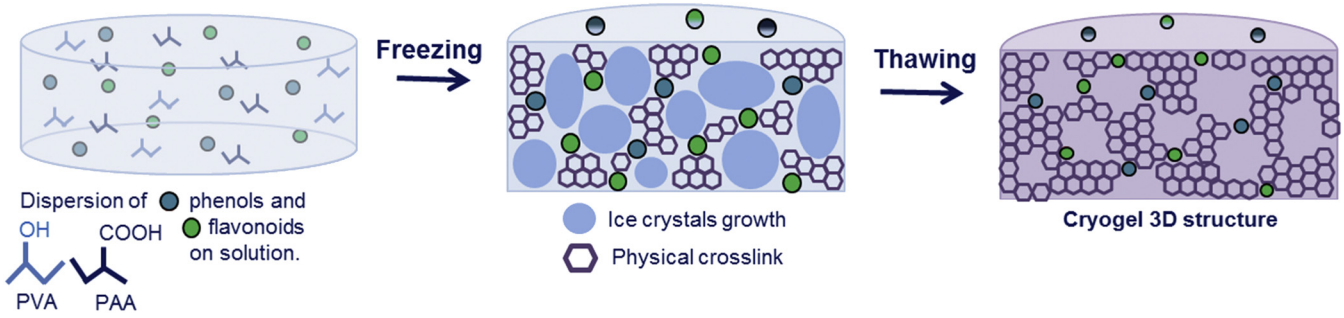

Figure 1. Synthesis of propolis-loaded cryogels.

Water retention studies were carried out after samples reached equilibrium swelling. The samples were dried in a vacuum oven at 60°C until the weight of the sample was constant. The water retention was calculated using the following formula:

$$W_r(\%) = \frac{W_0}{W_{ex}} \times 100, \quad (2)$$

where W_r (%) represents the percentage weight of the hydrogel at any specific time, also known as water retention, W_{ex} is the final weight of the sample after swelling and drying, and W_0 is the initial weight of the sample before the experiment.[34]

Propolis dissolution profiles were obtained using a Sotax AT7 smart dissolution system from Carl Stuart Ltd. Cylindrical shaped propolis-loaded hydrogels were (r = 17.5 mm, h = 18 mm) tested in phosphate buffer solution of pH 7.4 at 37°C. The stirring rate was set to 100 rpm with 900 mL of dissolution media used per vessel. Six vessels were used for each scan. After filtration, samples were automatically taken at set intervals and analyzed by ultraviolet (UV) light on a Perkin Elmer lambda 2 spectrometer. The percentage propolis release was determined using a standard calibration curve of ultrasound-assisted ethanol propolis.

Mechanical Properties

Rheological measurements were carried out using an AR 1000 rheometer from TA instruments. The tests were performed using the parallel plate method with a 20-mm steel plate geometry. Low frequency and low strain range was adopted. A strain sweep was applied from $1.8E^{-4}$ to $1E^{-3}$ at a frequency of 1 Hz. Frequency sweep was applied at a range of 0.1-100 Hz. In all cases, a compression load of 2 ± 0.5 N was exerted on the swollen hydrogels during testing. All data are presented as mean of 2 measurements.

Bacteria Strains

Sterile nutrient broth (100 mL) was inoculated with *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* IMD 121, or *Pseudomonas putida* IMD 135 and incubated at 37°C for 8 h until absorbance at 600 nm was in the range of 0.7-0.8. The bacteria were used at a concentration of 10^8 cfu/mL. Each culture was streaked on nutrient agar. In each agar plate, 3 wells were punched using a sterile bore, 10-mm diameter. Hydrogel samples were transferred into separate wells and covered with 50 μL of PBS (pH 7.4) to facilitate sample swelling. For each condition, 2 cultured plates were used. The samples were incubated for 24 h at 37°C. The zones of inhibition formed around the samples were measured, and antimicrobial properties of the cryogels were determined.

Bacteria Growth Curve

A growth curve was prepared for each bacterial strain used in the study. Briefly, 150 mL of nutrient broth was inoculated with 1.5 mL of liquid culture of different microorganisms, and incubated at 37°C with 100 rpm shaking. Aliquots (5 mL) were taken at different time intervals, and the optical density was measured at 600 nm, using sterile nutrient broth as a reference sample. The study was carried out in duplicate, for all four bacterial strains.

Results and Discussion

Extensive results already exist for ethanol-extracted propolis.[35,36] It is also important to note that propolis composition depends on the season and on the source from which the bees collect the resin, thus may interfere with biological activity.[37,38] Visual observation revealed the presence of unwanted wax after MAC, even after filtration, which was indicated by the occurrence of yellow particles.

Chemical Analysis of the Propolis Extracts

Propolis was extracted in 70% ethanol as it has been previously shown to be an effective method when extracting the main components of propolis.[25,39] Moreover, some authors stated that ethanol-based Soxhlet resulted in higher yields compared to extraction with other solvents.[40]

UV-VIS spectra of the different propolis extracts are shown in Figure 2 and compare well with other studies.[25,41,42] Samples obtained by SOX had the highest values of absorbance compared to SON and MAC.[42] Because of different extraction methods, Soxhlet presented a large baseline peak at 295 nm. Phenolic compounds generally exhibit an absorption peak in the ultraviolet light range of 250 and 350 nm for spectrophotometric analysis.[43]

MICs of Ethanol Extracts of Propolis

MIC was characterized as the lowest concentration of the samples that exerted a bacteriostatic effect. Although minimum bactericidal concentration (MBC) was defined as the lowest concentration in which the samples exhibited a bactericidal effect, namely, it reduced the initial population of test organism by 99.9% after 12 h of cultivation. Table 1 summarizes the MIC and MBC as described by Andrews (2001)[32] of the ethanol extracts of propolis (EEP) samples. The results show that MIC varied in different microorganisms and that the growth of gram-negative bacteria such as *E. coli* and *S. typhimurium* was only inhibited when higher concentrations of propolis were used. MBC for *S. aureus* was significantly lower than in other bacterial strains under investigation. Such results are in agreement with previous studies

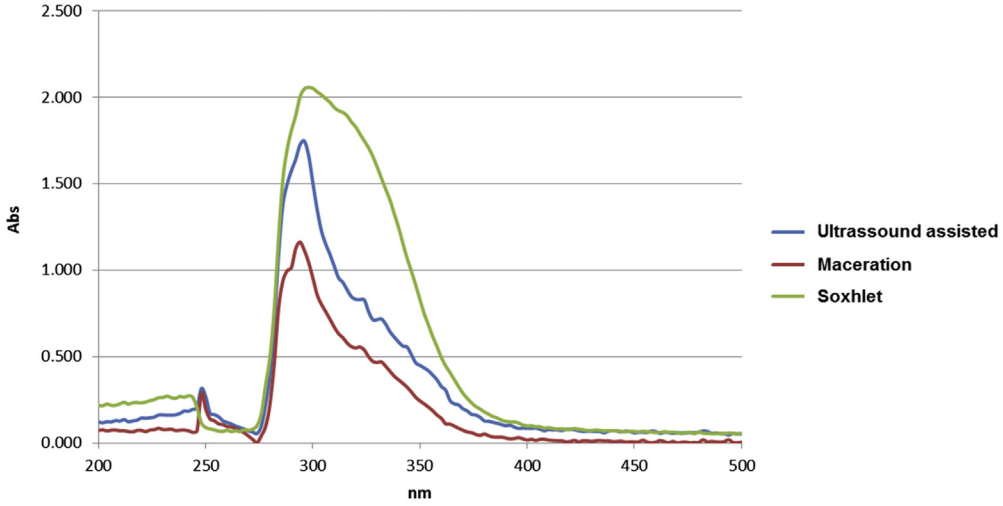

Figure 2. UV-VIS spectra for ethanolic-extracted propolis.

which show a strong bactericidal effect of propolis against gram-positive bacteria, while limited effect against gram-negative microorganisms.[21,38,44]

Microstructural Analysis

Each propolis extraction was mixed in a polymer matrix with different concentrations before 10 cycles of freeze and/or thawing. The objective of FTIR was to investigate the characteristic chemical bands of PVA and propolis on each group sample. FTIR of PVA, PAA, and propolis has been reported in literature.[10,30,45-47] FTIR characteristic bands and vibration modes from the literature[44,48] are displayed in Table 2.

Characteristic peaks of PVA were found in all samples. Polyvinyl alcohol and polyacrylic acid (Fig. 3a) peaks generally correspond to alcohol and carboxylic acid groups. The large band between 3500 and 3200 cm^{-1} was due to the stretching O-H.[45,49] With addition of PAA peaks at 1644, 1417, 917 cm^{-1} became more defined and exhibited a negative shift in spectra position which indicating the presence of hydrogen bonding as compared to pure PAA bands,[50,51] where these bands were not observed. Figure 3 shows the representative spectra of ultrasonic ethanol-extracted propolis and PVA hydrogels.

No significant changes can be observed for different EEP samples (Fig. 3a). SON obtained the most intense peaks from the studied extracted propolis, with bands at 2916 and 2853 cm^{-1}, attributed to increasing levels of aromatic compounds. The peaks at 1643 cm^{-1} correspond to the stretching of carboxyl groups and aromatic ring bands.[44] Nonetheless, Soxhlet EEP samples exhibited a reduction in peak intensity in comparison to other EEP methods.

For propolis-loaded samples with different concentrations (Fig. 3b), the peaks appears more defined and with increased intensity, owing to the increase in propolis concentration. With the addition of PAA (Fig. 3c) to the composites, the intensity of most of the peaks reduced in comparison to PVA-only samples. Moreover, for 50% propolis, a peak is formed at 1706 cm^{-1} and is associated with C=O stretching.[52] Increasing the concentration of propolis slightly reduced the C=O peak along and increased the intensity of the aromatic ring peaks at 1635 cm^{-1}.

Table 1
The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of Ethanolic Extract Propolis Against Gram-Negative and Gram-Positive Bacteria

Microorganisms	MIC (mg/mL)[a]	MBC (mg/mL)[b]
S aureus ATCC 25923 (n = 10)	0.43	1.50
P putida IMD 135 (n = 10)	0.43	2.33
E coli ATCC 25922 (n = 10)	0.67	2.33
S Typhimurium IMD 121 (n = 10)	1.00	2.33

[a] MIC was defined as the lowest concentration of EEP in which the bacteria cultured after 12 h were lower than the initial population or without significant difference ($p > 0.005$) from each other by the least significant different test.
[b] MBC was defined as the lowest concentration in which it could reduce 99.9% of the initial population.

Swelling Kinetics and Propolis Release

Swelling, propolis release, and gel fraction were investigated at pH 7.4 in buffered solution to understand the effects of propolis on these parameters (Fig. 4). In the case of swelling, samples containing PVA and propolis (Fig. 4a) start to exhibit a constant swelling rate at 10 h indicating that this hydrogel has rapid swelling characteristics. From the period studied (24 h), samples swelled to 350% and compare well with other studies where these hydrogels have been described as superabsorbent.[53] In addition, propolis-loaded samples appear to have a decreased swelling rate when compared to pure PVA, demonstrating a significant decrease ($p < 0.05$) in water absorption with increase in propolis loading. This may be due to the fact that the propolis is increasing the intramolecular bonding. Alternatively, this may be due to the fact that PVA/PAA hydrogels containing propolis (Fig. 4c), have a different molecular configuration compared to PVA alone. The swelling rate values are higher than those of PVA only and continue to increase even after 24 h. This increased swelling appears to be indicative of the pH sensitivity of the acrylates in PAA that is facilitated by the carboxylic acid groups.[54] Furthermore, higher concentrations of propolis resulted in a decrease in the swelling ratio, which may be attributed to the increased intramolecular bonding observed in the FTIR spectroscopy.

Extensive studies already have reported on drug release mechanisms in 3D hydrogels.[1,55,56] As a hydrogel swells, the mesh size enlarges allowing an encapsulated drug to diffuse out of the

Table 2
FTIR Bands Present in Each Original Sample

PVA (cm^{-1})	PVA Groups Vibration Modes	Propolis	Propolis Groups Vibration Modes
3302	Alcoholic—O-H stretching	3319	Stretching (OH) groups
2948	Stretching (C-H)—alkyl groups	–	
2909		2921	C-H bands of aromatic compounds
2850		2849	
–	–	1694	Stretching of carboxyl groups
1644	Stretching (C=O) of acetated groups, stretching of (C=C)	1634	Stretching (C=C), aromatic ring bands
–	–	1603	Aromatic ring bands
–	–	1515	
		1452	
1417	Bending, in plane (C-H in CH_2 groups); stretching (C-O-C), of unhydrolyzed acetate groups, in plane (O=H)	1405	C=C ring stretching occurs in pairs at 1638 and 1409
1378	Coupling of in plane (0-H) wagging (C-H)	1376	
1331	Bending (CH + OH), fan and twist ($-CH_2-$)	1331	
–	–	1263	C-O groups of polyols
1144	Stretching (C-O-C), stretching (C-C) crystalline sensitive band	1154	
1094	Stretching (C-O), bending (O-H)	1076	Stretching (C-O) of ester groups
–	–	1033	
917	Stretching (bending out of plane) (C-H)	–	–
–	–	861	Aromatic ring vibration
836	Stretching pendular (C-C)	835	
–	–	818	Aromatic ring vibration
–	–	777	
–	–	718	

gel. Because the gels in this research are partially swollen on synthesis, the drug is free to diffuse without any further swelling. Propolis release from polymers generally occurs by burst release of propolis in the first day of swelling. However, this is not always the case and prolonged release can also be achieved.[30] Because of the nature of the phenolic-rich compounds in the propolis, compound solubility varies with solution pH and has been shown to increase with increasing pH values due to dissociation of ionic bonds.[57]

Water retention tests (Figs. 4b and 4d) revealed an increase in water retention with increase in propolis concentration in the hydrogels. In contrast, the PVA gels containing propolis had the opposite effect, in that shrinkage was observed. The hydrogels contracted within minutes and during the first hour released

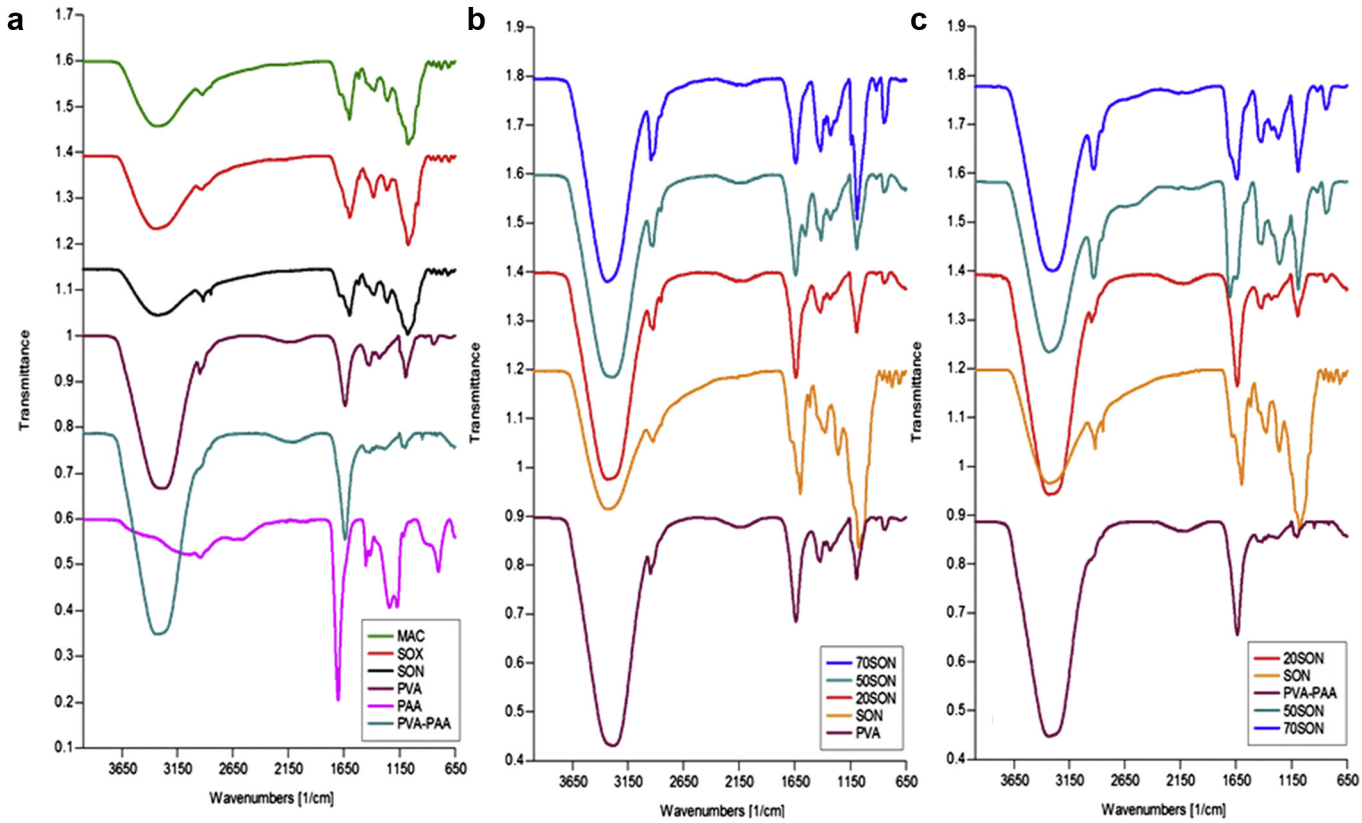

Figure 3. FTIR spectra of the original samples (a), PVA + propolis (b), and PVA + PAA + propolis samples (c).

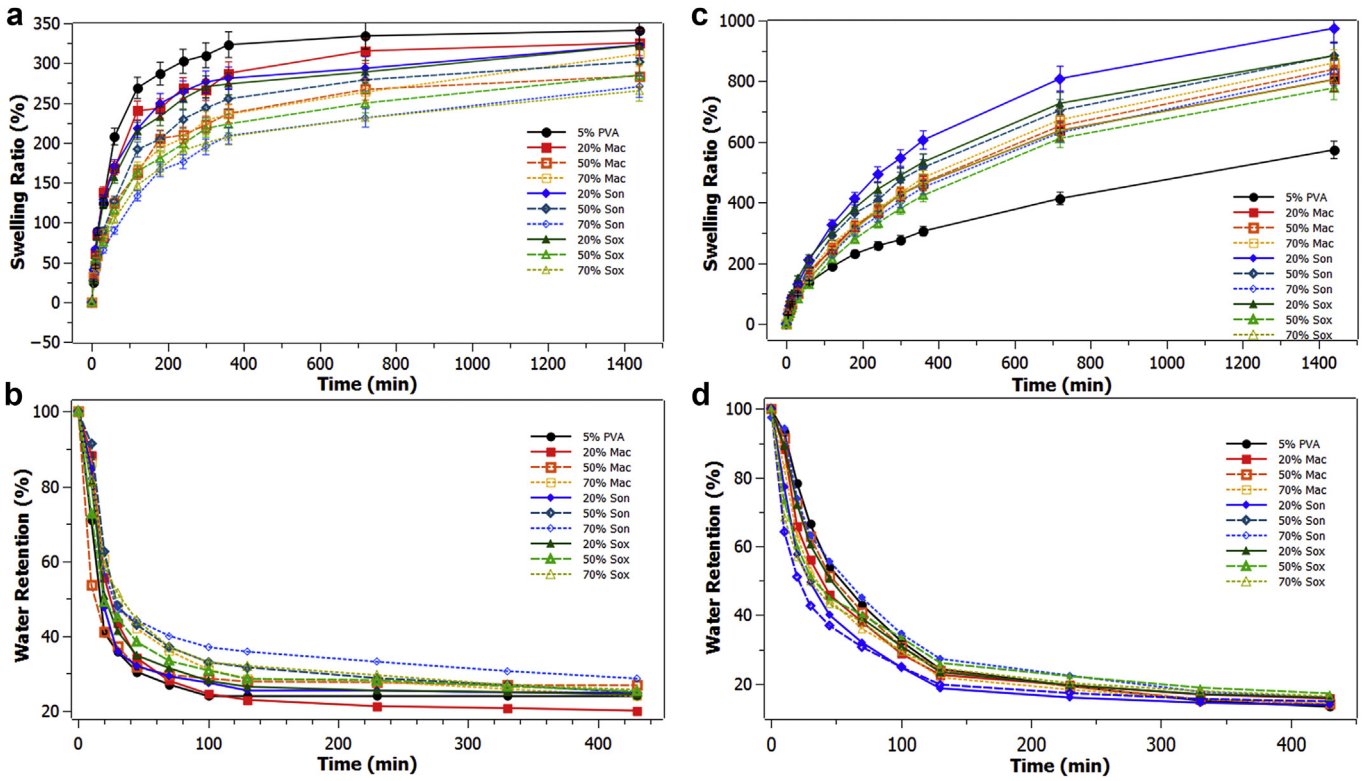

Figure 4. PVA samples (a) swelling and (b) water retention profiles; PAA samples (c) swelling and (d) water retention profiles of the studied samples.

50%-60% of water. With the addition of PAA, water retention increased and reached equilibrium after 5 h for most samples. These results are similar to the swelling data for the PAA hydrogels, where higher propolis content resulted in reduced swelling because of intramolecular bonding with water.

Propolis release studies (Fig. 5) for SON have a similar profile to their swelling rate, where a decreased release rate was observed with increased propolis concentration. Samples with 20% of propolis released the active ingredients of the compound faster. However, for samples with higher concentrations of propolis, a slower release rate was observed. For samples containing PAA, the values increased in comparison to PVA only. However, on highest concentration (70%), no significant effect on PAA was observed on release of the propolis. This effect might be explained by the increased intensity of the aromatic ring and C=O stretching of PAA bands seen in the FTIR analysis.

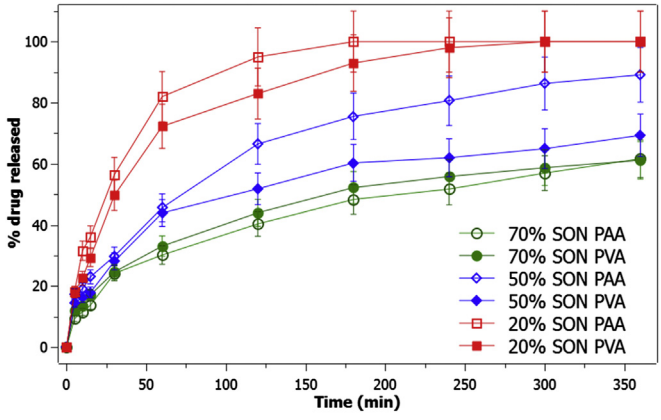

Figure 5. Drug release studies of ultrasound-assisted extracted propolis on PVA and PAA.

Mechanical Properties

The average storage modulus for each hydrogel was determined, and the results are shown in Figure 6a. The results of PVA are only shown because the PVA/PAA matrices had similar profiles but with lower values. With the addition of EEP, SON and SOX had different values as compared to MAC, but no statistical differences were detected. In contrast, values for maceration samples increased with increased concentration of propolis. This may be attributed to the fact that a significant amount of unwanted wax was observed after MAC and correlates with other studies where low molecular weight wax has been shown to act as a lubricant, thereby increasing flexibility.[36]

In support of the storage modulus values, frequency sweep data exhibited (Fig. 6b) a similar profile; MACs obtained the lowest value for the 20% propolis hydrogels and a rapid increase was observed with increasing concentrations up to the highest values for 70% of EEP. Soxhlet and ultrasound-assisted extractions had their values approximated to pure PVA.

Although statistical analysis reported no significant difference between samples, this could be related to the low “n” numbers. Nonetheless, the results presented here indicate a slight decrease in mechanical properties for pure PVA hydrogels for SOX and SON. This correlates well with data previously reported in literature by McGann et al.[58] Where a decrease in mechanical properties was observed for theophylline-loaded PVA and PVA/PAA because the theophylline interacted with the C=O groups on the PAA hydrogels. Furthermore, frequency-sweep analysis indicated that the composites behaved more like a solid because values recorded for the storage modulus were higher than corresponding loss

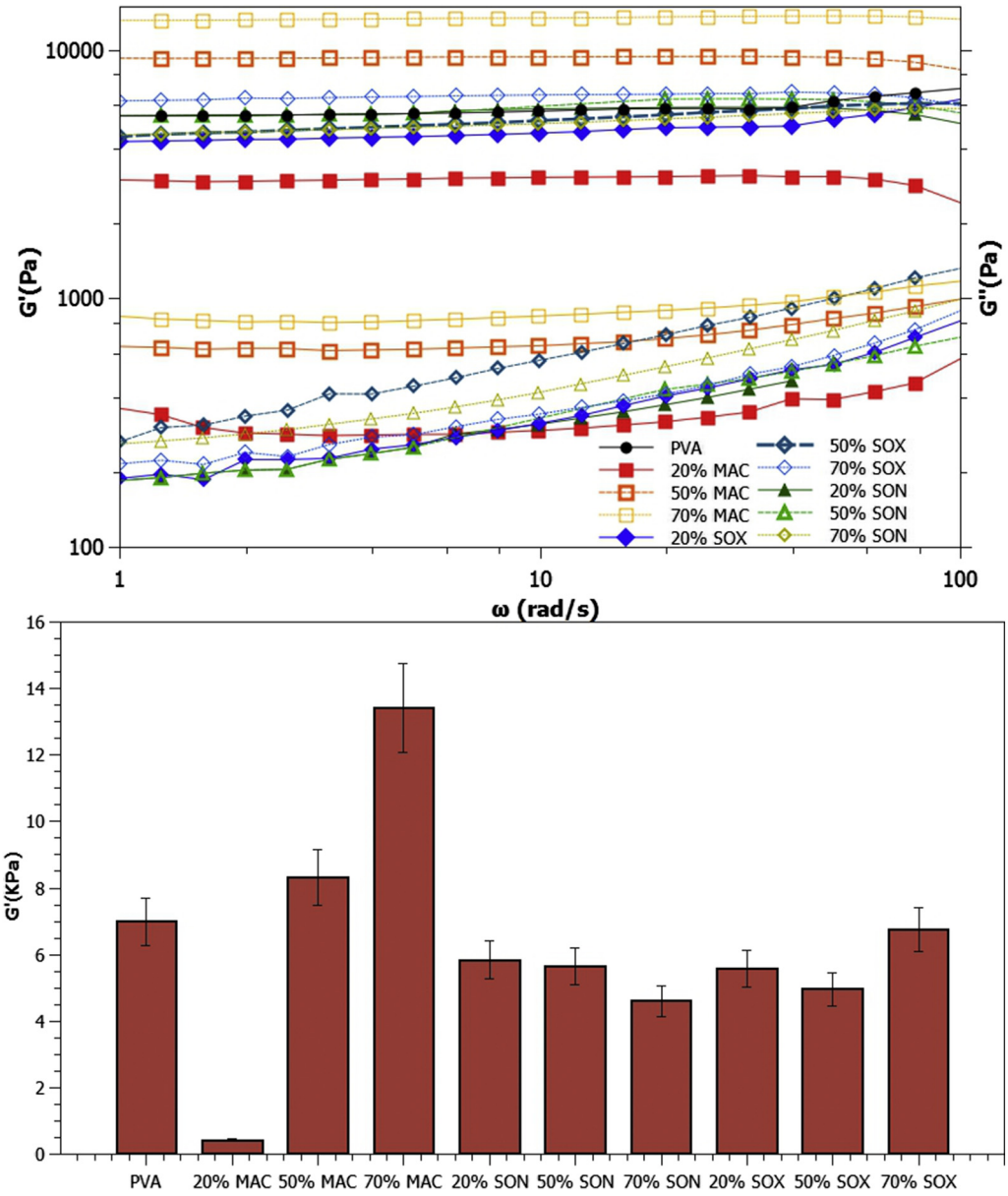

Figure 6. (Top) Strain-sweep tests from composites with and without propolis. (Bottom) Frequency sweep tests for the studied samples.

modulus values. This is a characteristic feature of cross-linked hydrogel.[59]

Antimicrobial Tests

The antimicrobial effect of PVA cryogels loaded with propolis is not well reported in the literature; therefore, this study was performed to evaluate the effect of different propolis extracts on various types of bacteria. Antimicrobial tests revealed that the inhibitory effect of cryogels loaded with different propolis extracts varied with different bacteria. The diameter of the zone of inhibition around ultrasonic EEP-loaded PVA cryogels (Fig. 7) was directly proportional to the concentration of propolis in the sample. The propolis extract obtained by SON was found to be very effective against *P putida* demonstrating the largest zone of inhibition. Maceration EEP-loaded PVA cryogels demonstrated inhibition of all 4 types of bacteria tested, but it was the least effective when compared to other extractions. Finally, Soxhlet extraction was only effective for one concentration. A direct statistically significant relationship was found between the concentration of propolis, method of extraction, and diameter of zone of inhibition ($p < 0.03$). Recent studies[30] reported that propolis exhibited no inhibition activity against *E coli*. However, the EEP studied in this work was very effective against *E coli*, but it should be noted that the region where it was collected had an effect on the antimicrobial properties.

PVA/PAA hydrogels loaded with propolis were also effective against the bacteria used in the study. However, these hydrogels presented reduced values for the diameter of the zones of inhibition compared PVA only. This might be due to the intramolecular bonding observed in the FTIR spectroscopy, which in turn may have prevented and/or slowed propolis release resulting in a reduced zone of inhibition. The effectiveness of propolis released from the PAA hydrogels on different bacteria was lower than that of propolis in the PVA samples where smaller diameters of the zones of inhibition were observed.

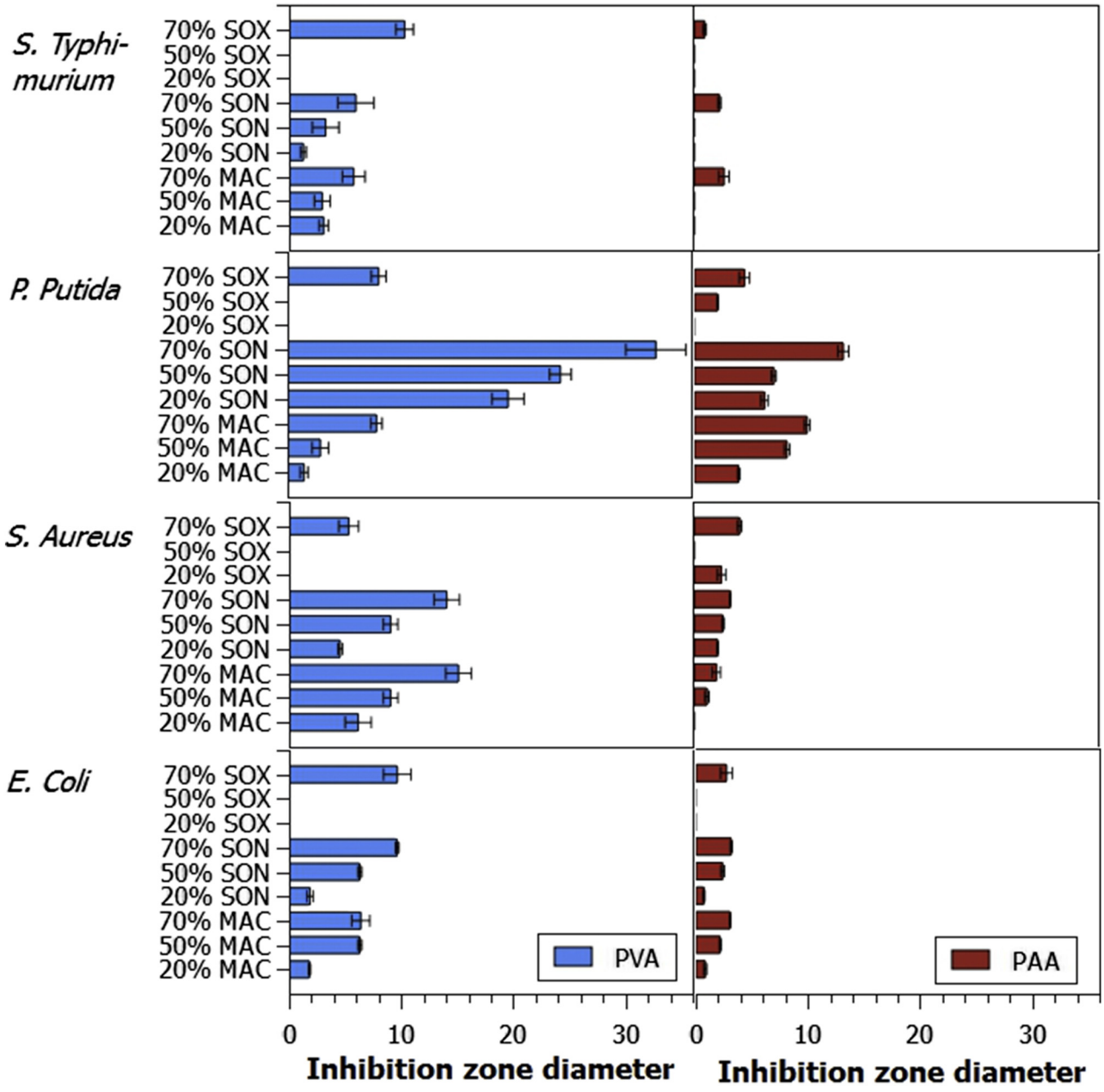

Figure 7. Antimicrobial activity of the PVA + propolis (blue) and PVA + PAA + propolis (red) samples against different bacteria. Significant differences were observed on all samples ($p < 0.03$) (n = 6).

MIC values of pure propolis indicate higher values than for samples with PVA-loaded propolis, suggesting that the latter preparation was more effective against bacteria. This may be related to the sustained release of propolis seen in the drug release studies and may have prevented bacteria from effectively metabolizing the propolis. As a consequence, these results suggest that samples with higher concentration of propolis can be considered for bactericide dressing.[29]

To understand the effect of Soxhlet-extracted propolis on bacterial activity, different concentrations of propolis were extracted by Soxhlet, propolis-loaded PVA hydrogels were prepared, and the effect on bacterial growth inhibition was evaluated. The results (Fig. 8) indicated that only the 70% propolis-loaded hydrogels had an inhibitory effect on bacterial growth. For further reference, the zones of inhibition on the plates for the different bacteria are listed on Figure 8b. Interestingly, the bactericidal effect of propolis was still observed after 9 h of incubation for *P putida* and *E coli*. Furthermore, a statistically significant decrease in *P putida* growth rate was observed for all concentrations of PVA containing Soxhlet-extracted propolis ($p < 0.03$).

Conclusions

In this work, the effect of different methods of propolis extraction, including MAC, SON, and SOX were examined with the aim of creating PVA and PVA/PAA hydrogels with varying concentrations of the extracted propolis. The incorporation of propolis in the hydrogels was confirmed by FTIR, where the aromatic ring bands of the propolis increased with increasing the concentrations of propolis. A decrease in hydrogel swelling was obtained with increasing concentration of

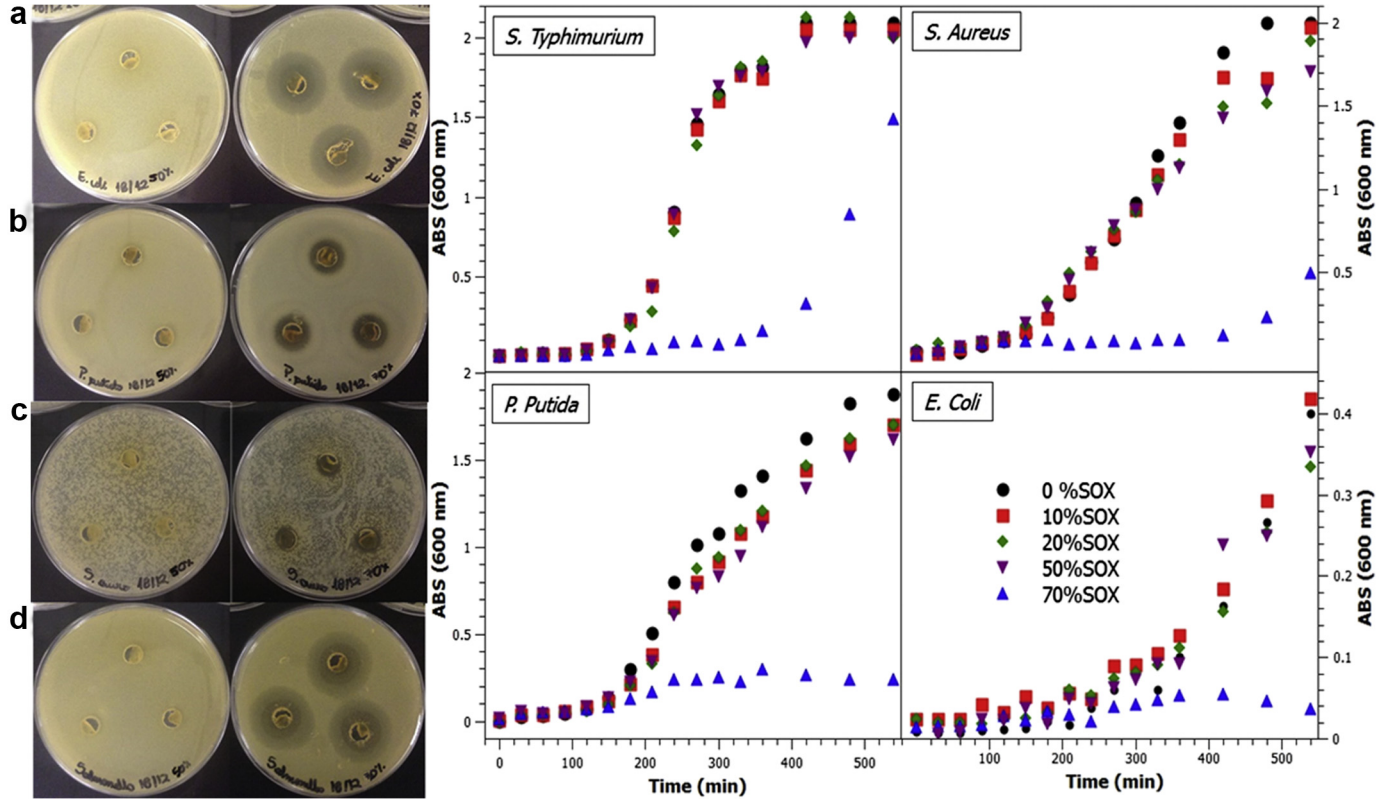

Figure 8. Inhibition zone diameter of PVA + (50% and 70%) Soxhlet samples on (a) *E Coli*, (b) *P Putida*, (c) *S aureus*, and (d) *S Typhimurium* and liquid culture bacteria growth profiles with contact of PVA + Soxhlet samples.

propolis, which in turn resulted in a reduction in propolis release. On the other hand, the addition of PAA to the hydrogel improved swelling and water retention as a result of the pH sensitivity. In terms of mechanical properties, propolis-loaded PVA hydrogels obtained different attributes for different extractions and apart from MAC; no major differences were observed between groups ($p > 0.05$). Antimicrobial studies revealed the highly effective inhibition of all bacteria studied. Moreover, bacterial inhibition increased with increasing concentrations of propolis. Ultrasound-assisted EEP proved to be the most efficient method of extraction for inhibiting the bacteria used in this study. In comparison, Soxhlet EEP appeared to be effective at one specific concentration. The addition of PAA to the hydrogel did not improve the antimicrobial properties, as bacterial inhibition was inferior to PVA alone. These results revealed that the efficacy of propolis can be enhanced by incorporation into hydrogel carrier systems and that PVA hydrogels with higher concentration of propolis may be considered for use as bactericide dressing.

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