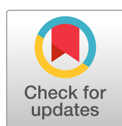

Design and *in vitro* antibiofilm activity of propolis diffusion-controlled biopolymers

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Abstract

In this study, a novel pH-sensitive hydrogel beads that is based on gelatin/sodium alginate/chitosan (GEL/SA/CS) loaded with propolis ethanolic extracts (PE) were synthesized. The swelling behavior of GEL/SA/CS hydrogel beads was studied in different pH solutions and compared with unloaded CS (GEL/SA) hydrogel beads. The *in vitro* release studies have been revealed using four different pH (1.3, 5.0, 6.0, and 6.8), a saliva environment (pH 6.8), a simulated gastric fluid (SGF) (pH 1.3), and a simulated intestinal fluid (SIF) (pH 6.8) to simulate the physiological conditions in gastrointestinal (GI) tract. Propolis-loaded hydrogel beads were found to be stable at pH 1.3, 5.0, 6.0, simulated saliva, SGF, and SIF mediums, whereas the beads lose their stability at pH 6.8 buffer solution. Tested microorganisms displayed greater sensitivity to PE-loaded hydrogel beads compared with pure propolis. Contrary to antimicrobial activity results, antibiofilm activity results of PE-loaded GEL/SA and GEL/SA/CS hydrogel beads were found at low levels. According to the obtained results, the propolis-loaded GEL/SA/CS hydrogel beads synthesized within this study can be used in the treatment of GI tract diseases such as oral mucositis, gastric ulcer, ulcerative colitis, and GI cancer, as controlled releasing carriers of propolis. © 2020 International Union of Biochemistry and Molecular Biology, Inc.
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Keywords: *antibiofilm, antimicrobial, biopolymer, drug carrier, gastrointestinal tract, propolis*

1. Introduction

Gelatin (GEL) is denatured collagen that contains polypeptide chains. GEL has been widely used in wound dressing materials and slow release systems. It has film-forming properties but it is rarely used alone due to its low intensity and high brittleness. Therefore, GEL is often used after modifications through several methods, such as cross-linking, grafting, and blending [1]. Alginate is a well-known polysaccharide and it is also possible to obtain an alginic acid gel by lowering the environmental pH value. Due to biocompatibility, mucoadhesion, porosity, and ease of manipulation properties of alginate calcium gels, they have recently been preferred for the delivery of proteins, cell encapsulation, and tissue regeneration. These polysaccharides are normally present as sodium salts that are called sodium alginate (SA). SA forms an anionic polymer when it is negatively charged and begins to entangle and swell. As a result, alginate is often exploited as a controlled-release vehicle in drug delivery systems (DDS). In terms of drug/protein delivery, numerous applications of calcium alginate gel beads or microspheres have been proposed [2, 3].

Calcium chloride ($CaCl_2$) is one of the most frequently used agents to ionically cross-link alginate. However, it typically leads to rapid and poorly controlled gelation due to its high solubility in aqueous solutions [4]. The ion type was found to be effective on encapsulation efficiency and drug release rate media. Ca^{2+} and Fe^{+3} ions were used as cross-linker for drug-loaded SA composites containing different cellulosic structures [5, 6].

Chitosan (CS) has drawn much attention because of its good biocompatibility, biodegradability, film forming ability, bioadhesivity, antimicrobial activity, wound healing, low toxicity, and absorption-enhancing properties [7, 8]. Consequently, microsphere and hydrogel structures containing CS/SA have

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Abbreviations: *GEL, gelatin; SA, sodium alginate; CS, chitosan; GI, gastrointestinal; DDS, drug delivery systems; PE, propolis extract; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; OD, optical density.*

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been used in different applications such as drug release systems and wound dressing materials [9-16].

Water-soluble derivatives of CS have been used in combination with alginate to prepare Ca^{2+}-crosslinked hydrogel beads, which generally exhibit pH-sensitive and ionic-sensitive swelling and drug release properties [17]. Drug application to the gastrointestinal (GI) tract has difficulties due to variable pH values in each part of the system.

Due to strong acidity of gastric juice, drugs can be released primarily in this location when employing a proper pH-sensitive biopolymer as the drug delivery carrier. CS has been proven to be a good candidate owing to the protonation of amine groups when pH is low, which is favorable for the release of drugs. Targeted drug delivery to the stomach is extremely important for the treatment of local maladies such as gastritis, gastro duodenal ulcer, and gastric cancer [18].

Specific drug delivery into the colon is highly desirable for the local treatment of a variety of bowel diseases such as ulcerative colitis, amebiosis, and colonic cancer. This is also important for the local treatment of colonic pathologies, and systemic delivery of protein and peptide drugs [19].

Oral route is usually preferred for the sake of convenience and comfort. However, the DDS employed should prevent the bioactive agent from degradation and avoid drug release and absorption in the stomach (pH 1–3) until the system reaches the colon. CS is biodegradable by colonic bacterial flora; thus, it is a polymer commonly used for colon drug delivery [20].

Propolis is a resinous substance collected and transformed by bees from parts of plants [21]. To the plant resins, salivary secretions and enzymes are added and the product is used mainly for protection against insects, invading microorganisms, and in beehives repair [22]. Propolis has different chemical compositions according to the botanical origin [23]. Propolis has been used by humans since ancient times for treating diseases. The abundance of chemical compounds in propolis provides multiple biological activities that characterize different geographical samples, such as anesthetic, antimicrobial, antioxidant, anti-inflammatory, and more recently, antiproliferative and antitumor activity [24]. In addition, propolis has been reported to be nontoxic, safe, and able to show antimicrobial synergism when administered in combination with some antibiotic drugs [25]. Propolis is currently incorporated into a wide range of complementary healthcare products, including tinctures, throat sprays, lozenges, toothpastes, soaps, and so on [26]. Topical therapy based on propolis widely applied in otolaryngology; nasal sprays containing hydroglyceric or alcoholic extracts of propolis are currently in the market [27]. Alone or incorporated in another dosage form, ethanolic extract of propolis is commonly used in dental treatments, due to its safety and efficacy [28]. The application of propolis in food, however, is still limited because it is soluble in alcohol and has a strong taste and aroma [29]. Ethanolic extract is the main propolis dosage form utilized on therapeutics [25, 28, 30].

Recently, researches using propolis for biomedical applications such as propolis-incorporated bioadhesive systems for treatment of periodontal diseases [28], protective approaches for dental health [31, 32], wound healing potential of nanostructured lipid systems [33, 34], biomedical membranes, and topical use of propolis have gained great attention [27, 35, 36].

In this study, SA, GEL, and CS are combined with $CaCl^{2+}$ for the first time to create a new carrier matrix. Propolis, consisting a large number of organic compounds, was loaded into the carrier matrixes. It is aimed to distribute the propolis in the carrier matrix by using GEL in the formulation. The outer surfaces of the carrier beads were covered with CS to increase the antibacterial property, as well as to control the propolis release rate. For this purpose, we investigated the possibility that propolis-incorporated GEL/SA/CS hydrogels could pass through the GI tract stages (mouth and stomach) without degradation. Antimicrobial and antibiofilm activity analyses of the carrier system were also performed.

Highlights

- The pH-sensitive GEL/SA and GEL/SA/CS hydrogel beads were synthesized in order to enable propolis extracts to be transferred without degradation.
- The synthesized hydrogel beads contributed to the controlled release of propolis extract in the gastrointestinal tract.
- Hydrogel beads, especially in the GEL/SA/CS structure, exhibited higher antimicrobial activity against test microorganisms in gastrointestinal environments.

2. Materials and Methods

2.1. Propolis

The samples were collected from beehives in 2015–2016 in different phytogeographical places of Mugla, Turkey. Six samples were obtained from: Bodrum (BM), Milas (MIS), Koycegiz (KZ), Kavaklidere (KE), Marmaris (MS), and Fethiye (FE). Propolis samples, grated after cooling, was extracted for 72 h with ethanol/water (70/30, v/v) mixture at room temperature (1:10, w/v). The extract was filtered and dried. The obtained propolis ethanolic extracts (PEs) were then transferred into amber ointment jars and then stored at 4 °C for at least 24 h prior to the study.

2.2. Microorganisms

A total of seven reference strains, obtained from American Type Culture Collection (ATCC, Manassas, VA) were tested: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, and *Candida albicans* ATCC 10239.

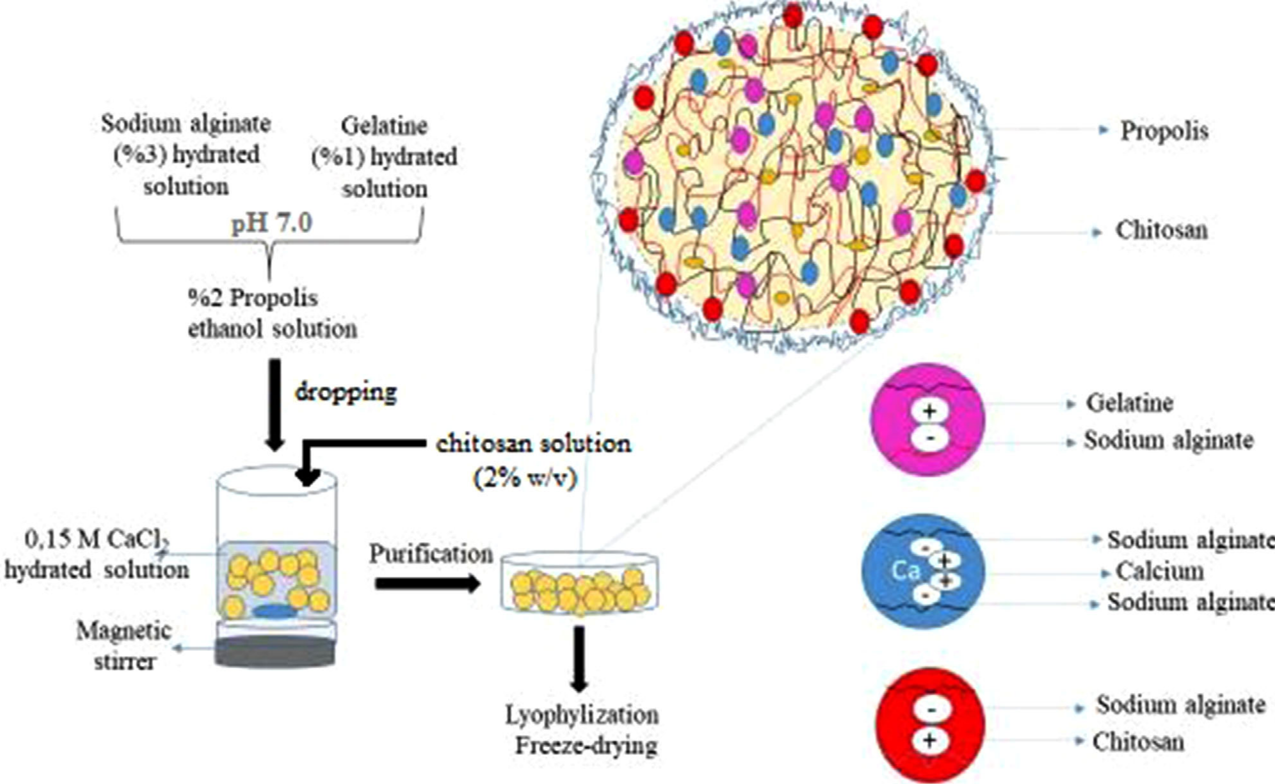

FIG. 1 *Scheme of the preparation of PE-loaded/unloaded pH-sensitive GEL/SA/CS hydrogel beads.*

2.3. Preparation of the PE-loaded/unloaded pH-sensitive GEL/SA/CS hydrogel beads

To prepare the GEL (1%, w/v) and SA (3%, w/v) solutions, GEL and SA powders were dissolved in acetic acid (1.2%, v/v) and distilled water, respectively. Both solutions are taken in equal volume and mixed and the final pH of the solution is adjusted to pH 7.4 with 0.1 N HCl. PE dissolved in 1 mL of ethanol was added to 4 mL of this solution by mixing and sonication.

This mixed solution was injected dropwise into $CaCl_2$ solution (0.15 M, pH 3) under 200 rpm constant stirring to get GEL/SA hydrogel beads. Homogeneous CS solution (2%, w/v) prepared in 2 mL of 1% acetic acid solution was added to this medium after 5 min by injection and mixing continued for 30 min to obtain CS-coated GEL/SA beads.

Similarly, beads without propolis can be obtained by removing the propolis addition step, and the CS-free beads by removing the CS addition step. After this stage, hydrogel beads were washed with 0.01 N acetic acid twice and distilled water in order to remove the foreign substances in the solution. PE-free/loaded hydrogel beads were then frozen at −80 °C and lyophilized using a freeze-dryer (Alpha 1–4 LSC plus; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) (Fig. 1).

2.4. Swelling measurements

The swelling behaviors of the dried hydrogel beads were measured at 37 °C using buffer solutions of pH 1.3, 5, 6, 6.8, and saliva (pH 6.8), a simulated gastric fluid (SGF) (pH 1.3), and a simulated intestinal fluid (SIF) (pH 6.8) with gentle agitation. At specific time intervals, the swollen hydrogel beads were withdrawn from the swelling media and blotted with a piece of paper towel to remove the excess water on the surfaces. The wet weight of swollen hydrogel beads was weighed on an electronic balance. Each experiment was repeated three times. The swelling ratios of hydrogel beads were determined using the following formula:

$$\text{Swelling ratio} = \frac{W_W - W_D}{W_D}$$

where W_W and W_D are the wet weight of the hydrogel beads and dried weight of hydrogel beads, respectively.

2.5. Release studies

The *in vitro* PE release studies of hydrogel beads were performed in a simulated GI tract conditions at 37 °C. Accurately weighed amounts of dried hydrogel beads (0.014 g) were placed in beakers containing 10 mL buffer solutions (pH = 1.3, 5.0, 6.0, 6.8) or saliva, SGF, and SIF. And then, the solutions were incubated at 37 °C with continuous agitation (130 rpm). At predetermined time intervals, 0.5 mL samples were collected from the release medium and replaced with fresh buffer

solutions. The amount of PE released from the hydrogel beads was assayed by UV spectroscopy at 420 nm. The percentage of cumulative amount of released PE was evaluated from standard calibration curves. *In vitro* release studies were repeated three times.

2.6. Determination of antimicrobial activities of PE-loaded/unloaded hydrogel beads

Antimicrobial activities of PE-loaded/unloaded hydrogel beads were evaluated using broth macrodilution method adapted from the document M07-A8 of Clinical and Laboratory Standards Institute (CLSI 2009) [37]. Briefly, 0.014 g hydrogel beads, the same amount used for release assays, were added to tubes containing Nutrient Broth. These tubes were inoculated with microorganisms by adjusting the concentration of 0.5 McFarland. Tubes without hydrogel beads were used as controls. After incubation, the turbidity of the medium (growth) was measured spectrophotometrically at 600 nm and % inhibition was calculated by the following formula:

$$\% \text{ inhibition} = \frac{OD_{Control} - OD_{Sample}}{OD_{Control}} \times 100$$

2.7. Determination of antibiofilm activities of PE-loaded/unloaded hydrogel beads

The antibiofilm activity of PE loaded/unloaded of hydrogel beads on test microorganisms was tested using a modified microplate biofilm assay [38]. PE and hydrogel beads containing same amount of propolis (0.014 g) were added to TSB test tubes with glucose 0.25%. Test microorganisms were inoculated onto the mixture and Dying procedure of Merritt et al. [38] was applied at the end of the 48 h incubation. Finally, optical density (OD) of each well was measured at wave length of 550 nm (Thermo Scientific Multiskan FC, Vantaa, Finland). Biofilm formation in the tubes containing only medium and bacteria was accepted as 100% and the antibiofilm rate of PE and propolis-loaded hydrogel beads were calculated according to the formula:

$$\text{Biofilminhibition } (\%) : \frac{OD_{Control} - OD_{Sample}}{OD_{Control}} \times 100$$

3. Results and Discussion

3.1. Preparation and characterization of blank and propolis-loaded hydrogel beads

Sodium alginate is negatively charged in aqueous solution, whereas pH is neutral above 7. Under these conditions, SA and GEL do not perform any cross-linking reaction. When the pH of this mixture is lowered, GEL is positively charged and immediately cross-links with the SA that is present in the medium. To have a more stable structural conformation, the particles are suspended in a medium that contains Ca^{+2}. Each of the Ca cations in the $CaCl_2$ structure is cross-linked by ionic interaction with an excess of the negatively charged alginate anions. CS is an antimicrobial substance with a positive charge. In the CS-added formulations, (+) charged ends of the CS ionically interact with the (−) charged ends of SA and form a layer in the outer regions of the beads. Thus, the PE-loaded GEL/SA matrix structure was coated with CS and a more stable hydrogel structure was obtained. There are studies in the literature where CS is used for this purpose and an increase in antimicrobial and antibiofilm activities is reported [39-41]. The codes and contents of the PE-loaded/unloaded pH-sensitive GEL/SA/CS hydrogel beads synthesized in the study are given in Table 1. The pH-sensitive CS-coated/uncoated GEL/SA hydrogel beads synthesized in the study are seen in white color. When PEs were loaded on these hydrogel beads, it was found that the hydrogel beads turned into yellow-orange shades depending on the chemical components of the PEs (Fig. 2). In addition, as seen in Fig. 2, the average particle sizes of hydrogels were evaluated as 1–2 mm diameters that are not monodispersed.

TABLE 1 *Code and formulations of PE-loaded GEL/SA and GEL/SA/CS hydrogel beads*

Origin of propolis extracts	*Bead codes*	*Chitosan*	*Propolis amount (g)*
Koycegiz	KZ-3	+	0.05
	KZ-5	–	0.05
Bodrum	BM-1	+	0.05
	BM-2	–	0.05
Marmaris	MS-2	+	0.05
	MS-3	–	0.05
Kavaklıdere	KE-1	+	0.05
	KE-2	–	0.05
Fethiye	FE-1	+	0.05
	FE-2	–	0.05
Milas	MIS-1	+	0.05
	MIS-2	–	0.05
Control	KL-1	+	0
	KL-2	–	0

3.2. Swelling studies

The swelling behaviors of GEL/SA (KL-2) and GEL/SA/CS (KL-1) beads at a SGF (pH 1.3), a SIF (pH 6.8), a simulated saliva fluid (pH 6.8), and at different pH solutions (1.3, 5.0, 6.0, and 6.8) have been determined.

According to the results of equivalent swelling rate of the samples at different pH buffer solutions and at GI tract, the highest value has been obtained for the carriers produced without loading propolis at pH 6.0 (Fig. 3). While the rate of swelling decreased in low pH, this ratio reached a maximum level at around pH 6.0. This unexpected result may be due

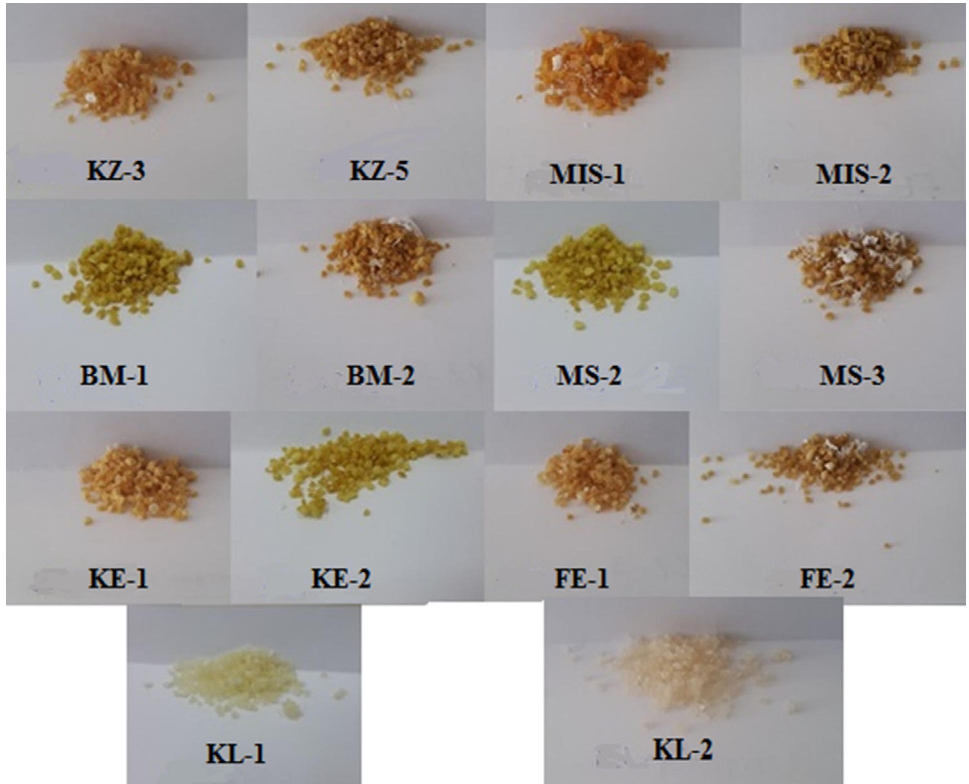

FIG. 2 *Digital photographs of PE-loaded/unloaded GEL/SA and GEL/SA/CS hydrogel beads.*

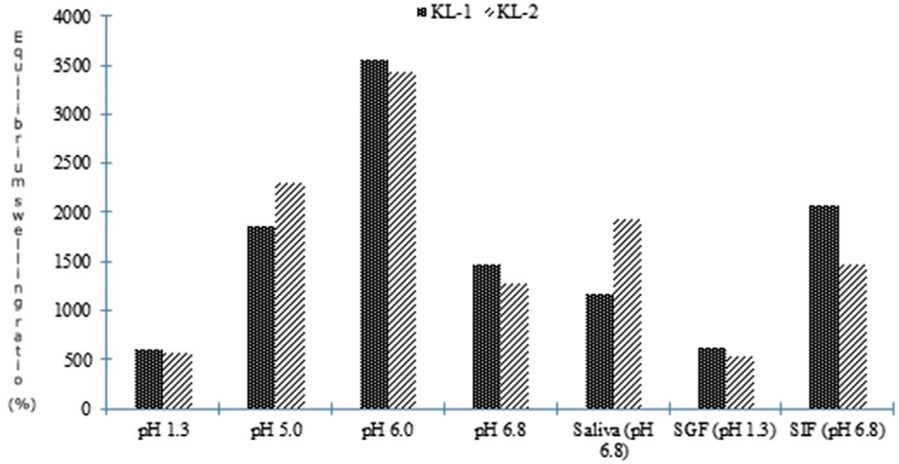

FIG. 3 *Swelling behavior results of GEL/SA (KL-2) and GEL/SA/CS (KL-1) beads at a simulated gastric fluid (SGF, pH 1.3), a simulated intestinal fluid (SIF, pH 6.8), a simulated saliva fluid (pH 6.8), and at different pH solutions (1.3, 5.0, 6.0, and 6.8).*

to the rapid disintegration at pH 6.8 and the reduction of the water absorption capacity of the remaining particles as a result of the separation of the polymer chains from the particle structure. The equivalent swelling ratios in saliva and SIF environments with a pH of 6.8 were higher than that of the SGF medium with a pH of 1.3. The reason why KL-1 and KL-2 formulations have different ratios in saliva and SIF medium is thought to be related to the differences in the chemicals used in the preparation. The results of equivalent swelling ratios in the GI tract environments with pH buffer solutions have shown similarity. Similar results have been reported for the same pH media and GI tract fluids [32, 35].

3.3. *In vitro* release of propolis from GEL/SA and GEL/SA/CS hydrogel beads

The PEs release of GEL/SA and GEL/SA/CS hydrogel beads was investigated at pH 1.3, 5.0, 6.0, and 6.8 buffered solutions depending on time (Fig. 4). In the release tests, only MIS-1 was released after 6 h from the hydrogel beads with the addition of CS in the medium at pH 1.3 and at the end of 24 h, a propolis release of 0.4 mg/g was detected. In the same pH environment, the hydrogel beads, which did not contain CS, were all released in the first hour. Propolis release was stopped in the MIS-2-coded hydrogel bead after 4 h. However, the KZ-5-coded bead was released at the maximum (1.6 mg/g) level at the 6th hour and the propolis release was over at the end of the 24th hour. In FE-2, MS-3, and BM-2 hydrogel beads, though the amount of propolis release was decreased after 6 h, the release continued until the end of the 24th hour.

In the other release medium set at pH 5, the highest propolis release was exhibited with KZ-3-coded beads containing CS. KZ-3 released 25 mg/g propolis at the 3rd hour, followed by decreased propolis release at the 6th hour, after which propolis release increased to 23.75 mg/g in 24 h. At the same pH medium, MIS-1 showed a regular release of propolis at 6 h and released propolis at the level of 9.13 mg/g in 24 h.

Propolis release rates of hydrogel beads without CS in the same pH environment were found to be lower than those in CS hydrogels. The highest propolis release in these hydrogel beads was KZ-5 at 4.54 mg/g in 29 h. It was followed by MIS-2 and BM-2-coded hydrogel beads.

In the pH 6 medium, among the propolis release rates of the carrier molecules containing CS, it was determined that the highest propolis release was in the KZ-3-coded hydrogel beads. KZ-3 showed the propolis releases at the level of 119.67 mg/g in 24 h and 136.75 mg/g in 30 h. Another carrier molecule that released propolis in this medium was MIS-1 and it was found that releasing ratios of this molecule were at 14.16 mg/g in 24 H and 18.39 mg/g in 30 h.

When the hydrogel beads without CS were evaluated in the same pH medium, MIS-2 reached the maximum level (29.41 mg/g) of propolis release at the end of the 1st hour and showed a stable release graph for 24 h.

Other hydrogel beads did not release propolis for up to 6 h, but KZ-5 and FE-2 hydrogel beads began to unleash after 6 h.

After 30 h, the KZ-5 molecule was released at 48.86 mg/g and the FE-2 molecule at a rate of 14.33 mg/g propolis.

In the pH 6.8 medium, propolis release was carried out by all of the CS hydrogel beads. Propolis secretion was detected for up to 4 h and propolis was depleted for release after the 4 h in all of the hydrogel beads. It was determined that hydrogel beads were disintegrated after 4 h in pH 6.8 and their physical structures were deformed. It was observed that hydrogel beads that release high amounts of propolis are deformed in a shorter time, whereas it was determined that BM-1 and MIS-1 hydrogel beads with low propolis release rate have a more stable structure. At the end of the 4th hour, the highest propolis release was measured as 143.09 mg/g in KZ-3 and 132 mg/g in FE-1.

In the pH 6.8 medium, the propolis release results of CS-free hydrogel beads were similar to those of CS-containing hydrogel beads. It was observed that the hydrogel beads had discontinued the release of propolis at the end of the 4th hour and breakdowns in their physical structures were occurred. In the first 1.5 h, the highest propolis was released by KZ-5 and after that time by FE-2. Propolis release at 4 h was measured as 149.2 mg/g for FE-2 and 112.92 mg/g for KZ-5.

The release profiles of PEs from hydrogel beads in simulated GI fluids are given in Fig. 5. In the simulated saliva (pH 6.8) medium, the highest propolis release in hydrogel beads containing CS was detected in MIS-1 (281 mg/g particle), with a higher rate than the pH 6.8 medium. This result showed that the buffering medium affects the amount of release. Although the swelling rates were the same level, the higher propolis release in the simulated saliva environment has shown that the chemical structure of simulated saliva is more suitable for releasing.

In the simulated saliva environment, KZ-3 was stable and achieved good propolis release. It was found that hydrogel beads without CS released very low amounts of propolis except MIS-2 reached 334.17 mg/g particle propolis release at the 4th hour and continued to release propolis for up to 24 h.

The highest propolis release in hydrogel beads containing CS in SIF medium was detected at MIS-1 at a rate of 61.07 mg/g in 6 h. Propolis release in MIS-1 was over 24 h. Propolis release was not detected in SIF medium in other carrier hydrogel beads. Propolis release was also detected in MIS-2, MS-3, and KZ-5 encoded hydrogel beads in non-CS carrier microbeads in SIF. It was determined that propolis release started at the 6th hour of MIS-2 and reached 15.56 mg/g particle level at 8th hour, and then there was a decrease in time-dependent manner. The KZ-5 released propolis at a particle level of 5.76 mg/g over a period of 0.5 h and then stopped to release. MS-3 produced lesser amounts, but showed a stable release of propolis.

In SGF medium, only MIS-1 was found to release propolis among the CS containing carrier molecules. At the end of the 3rd hour, MIS-1 was exposed to release propolis at the particle level of 465.27 mg/g. The high propolis release of MIS-1 in saliva medium with pH 6.8 was thought to be due to the similarity of the saliva media and the components of the SGF medium. At the end of the period, the amount of propolis measured as 0 mg, which indicates reabsorption.

In the SGF medium, the hydrogel beads showing propolis release was observed as MIS-2 between the hydrogel beads without CS. However, it has been found that these hydrogel beads release propolis at a lower level than CS-containing forms. In this medium, the release of propolis was also carried out by FE-2 from the carrier hydrogel beads, which did not contain CS. Propolis release from FE-2 was terminated at the end of 1.5 h, whereas for MIS-2, especially after 5 h post release was reactivated.

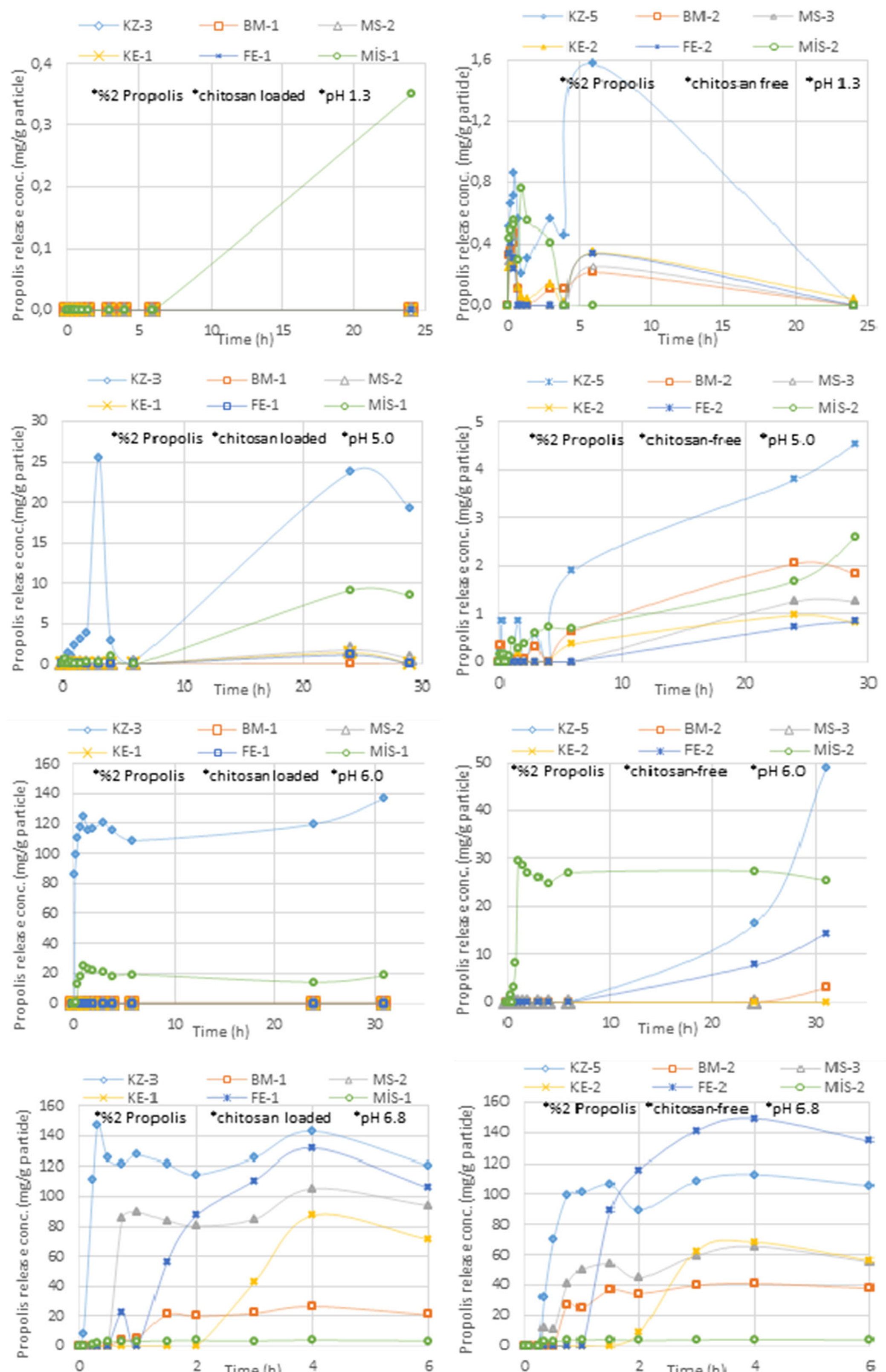

FIG. 4 *The release of propolis extracts from GEL/SA and GEL/SA/CS hydrogel beads in four different pH (1.3, 5.0, 6.0, and 6.8) environments.*

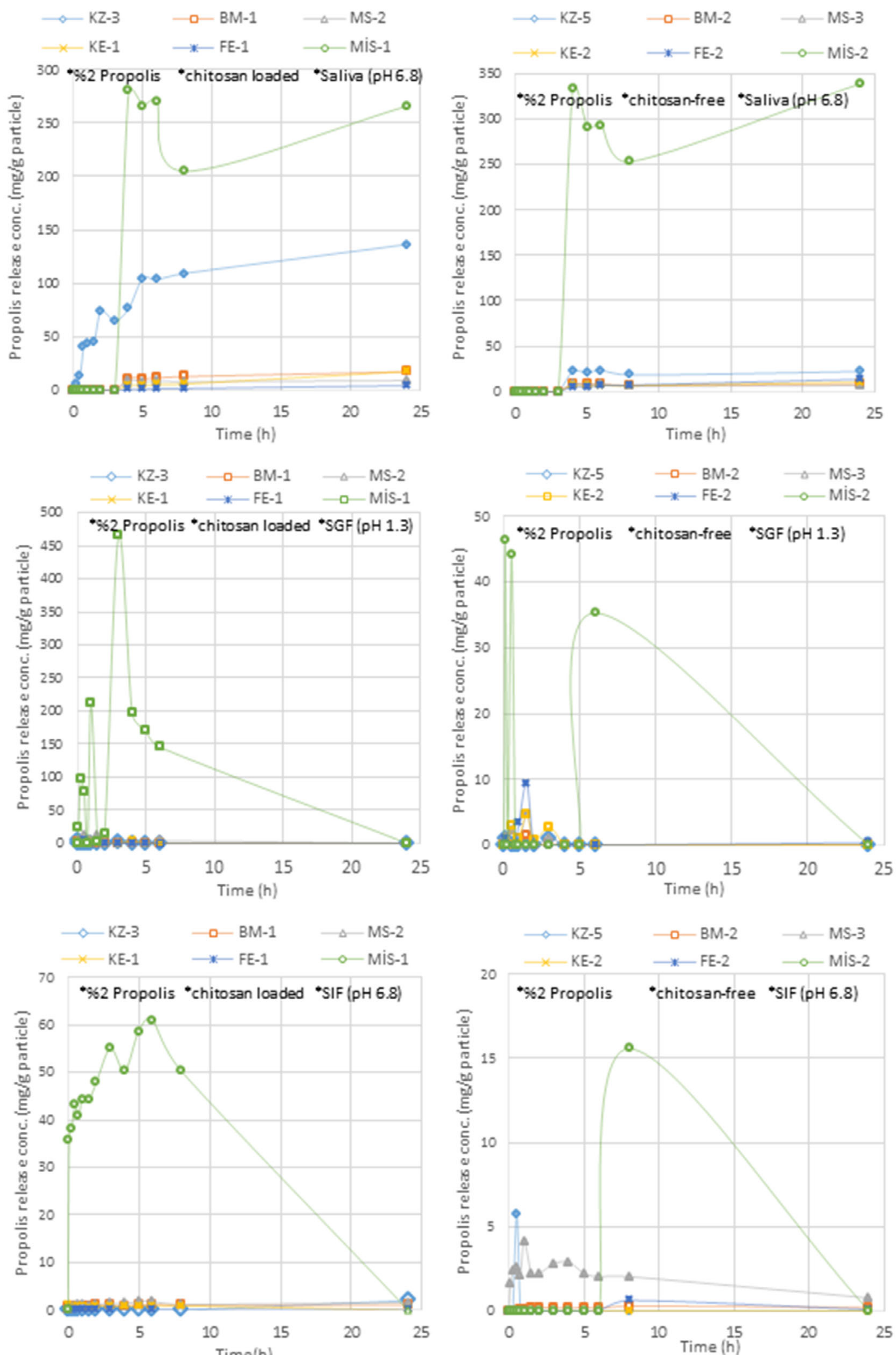

FIG. 5 *The release of propolis extracts from GEL/SA and GEL/SA/CS hydrogel beads in a simulated saliva, a simulated gastric fluid (SGF), and a simulated intestinal fluid (SIF).*

TABLE 2 *Antimicrobial activity results of PE-loaded GEL/SA and GEL/SA/CS hydrogel beads and pure propolis extracts*

M. organisms	B. subtilis *ATCC 6633*	S. aureus *ATCC 25923*	E. faecalis *ATCC 19433*	E. coli *ATCC 25922*	P. aeruginosa *ATCC 27853*	S. typhimurium *ATCC 14028*	C. albicans *ATCC 10239*
	% inhibition						
PE codes	Propolis extracts						
KZ	78.0 ± 0.2	37.1 ± 0.3	53.1 ± 0.5	24.0 ± 0.2	58.7 ± 1.3	43.8 ± 0.5	17.4 ± 0.5
BM	77.5 ± 0.4	68.6 ± 0.7	71.4 ± 1.2	63.8 ± 1.1	84.4 ± 2.1	88.7 ± 1.8	63.8 ± 1.3
MS	62.2 ± 0.2	76.2 ± 0.9	52.6 ± 0.5	60.3 ± 0.7	46.9 ± 0.7	69.1 ± 1.4	–
KE	100 ± 0	73.3 ± 0.3	28.2 ± 0.5	52.0 ± 0.5	48.3 ± 1	59.5 ± 0.5	25.1 ± 1
FE	100 ± 0	39.5 ± 0.5	68.3 ± 1.5	41.7 ± 0.3	48.7 ± 1.4	62.4 ± 1.6	12.4 ± 0.8
MIS	96.7 ± 0.5	74.7 ± 0.7	77.3 ± 0.8	26.7 ± 0.7	77.6 ± 1.5	100 ± 0	25.8 ± 0.9
Hydrogel codes	PE-loaded hydrogel beads						
KZ-3	89.0 ± 1.1	45.8 ± 0.3	93.0 ± 1.2	26.7 ± 0.5	–	95.9 ± 2	81.8 ± 2
KZ-5	92.3 ± 1.5	43.6 ± 0.5	95.3 ± 2.2	21.6 ± 0.7	–	96.9 ± 1.7	83.2 ± 1.2
BM-1	90.4 ± 1.7	27.6 ± 0.3	89.9 ± 1.8	10.8 ± 0.3	–	97.1 ± 1.3	71.9 ± 0.7
BM-2	73.2 ± 1.5	9.4 ± 0.4	24.2 ± 0.7	–	–	40.4 ± 0.8	91.6 ± 2.3
MS-2	82.8 ± 2.1	39.3 ± 0.5	77.8 ± 1.4	9.6 ± 0.2	2.8 ± 0.2	96.4 ± 1.5	90.4 ± 2.1
MS-3	44.5 ± 0.8	0.6 ± 0.1	21.6 ± 0.4	–	–	33.7 ± 0.7	88.7 ± 1.6
KE-1	50.7 ± 1	–	–	2.5 ± 0.4	–	26.9 ± 0.4	85.6 ± 1.1
KE-2	–	1.1 ± 0.2	–	–	–	10.3 ± 0.6	92.6 ± 2.5
FE-1	53.6 ± 0.8	9.8 ± 0.4	27.3 ± 0.9	3.1 ± 0.2	–	18.0 ± 1.1	87.0 ± 1.4
FE-2	64.1 ± 1.7	12.3 ± 0.3	10.0 ± 0.2	–	–	9.5 ± 1.2	94.0 ± 1
MIS-1	65.1 ± 1.8	14.8 ± 0.6	44.0 ± 0.9	–	5.0 ± 0.3	60.4 ± 1.7	89.9 ± 1.6
MIS-2	45.0 ± 0.8	10.4 ± 0.1	55.1 ± 0.7	–	–	75.4 ± 1.9	93.8 ± 1.2

–, no inhibition.

3.4. Antimicrobial activities of newly synthesized pH-sensitive hydrogel beads

Antimicrobial activities of PE-loaded/unloaded GEL/SA and GEL/SA/CS hydrogel beads were determined by broth macrodilution method against most commonly found bacteria, such as Gram-positive strains (*B. subtilis*, *S. aureus*, and *E. faecalis*), Gram-negative strains (*E. coli*, *P. aeruginosa*, and *S. typhimurium*), and yeast (*C. albicans*), and the results are given in Table 2. Antimicrobial effects of propolis in the amounts added to hydrogel beads against test microorganisms were determined as control. The strongest antibacterial activity was exhibited by KE and FE against *B. subtilis* with a 100 ± 0% inhibition. All PEs used in the study were found to be antimicrobial effective in the range of 62.2%–100.0% against *B. subtilis*. It has been determined that PEs also exhibited strong antibacterial activity against all Gram-positive bacteria. In particular, MIS inhibited *B. subtilis*, *S. aureus*, and *E. faecalis* at rates of 96.7%, 74.7% and 77.3%, respectively. A higher antimicrobial effect against Gram-positive strains has been reported in previous propolis antimicrobial activity studies [39, 42-45].

The antimicrobial activity results of PEs revealed that BM and MS had moderate antibacterial effects against *E. coli*. On the other hand, BM and MIS had high antibacterial effects against *P. aeruginosa* and *S. typhimurium*, respectively. The highest antifungal activity was detected with 63.8% inhibition in the BM against *C. albicans*. Although it has been demonstrated in many studies that propolis contains antimicrobial effects, it is difficult to compare because it contains different chemical compositions

and/or different antimicrobial activity tests [46]. Similar to many studies reported previously, our PEs were found to have higher antibacterial effects against Gram-positive bacteria than Gram-negative bacteria [47-49].

The antimicrobial effects of KZ, BM, and MS loaded GEL/SA/CS hydrogel beads have increased especially against *B. subtilis, E. faecalis*, and *S. typhimurium* compared with the antimicrobial effects of the same amounts of these extracts. Among the pH-sensitive hydrogel beads, the highest activity was observed for BM-1 polymer against *S. typhimurium* as 97.1%. KZ-3, KZ-5, and MS-2 biopolymers also exhibited inhibition activity of more than 90%. On the other hand, PE-loaded hydrogel beads did not show any inhibition potential against other Gram-negative bacteria; *E. coli* and *P. aeruginosa*. Considering the Gram-positive bacteria, the highest antimicrobial activities were observed for KZ-3, KZ-5, BM-1, and MS-2 biomolecules against *B. subtilis* and *E. faecalis*. It was figured out that PE-loaded hydrogel beads had antimicrobial activity against *C. albicans*. Antimicrobial activity assay results indicated that hydrogel beads have higher antimicrobial activity potential than their corresponding PEs. It is thought to be effective in increasing this effect in hydrogel structure and CS, which is known to have antimicrobial effect in this structure. This effect was taken into consideration especially in the increase of antifungal effect in hydrogel structures against *C. albicans*. It was also observed that, GEL/SA/CS hydrogel beads have higher antimicrobial activity than GEL/SA hydrogel beads.

The increase of antimicrobial effects of hydrogel beads when loaded with propolis revealed that the combination of propolis and GEL/SA/CS had a synergistic effect. Especially, this antimicrobial effect increase was observed in our study against *B. subtilis*, *E. faecalis*, and *S. typhimurium*. This increase in effect observed by loading propolis into hydrogel structures was confirmed by published data [39, 50-51].

3.5. Antibiofilm activities of newly synthesized pH-sensitive hydrogel beads

The inhibition effect of PE-loaded pH-sensitive hydrogel beads against the biofilm formation of tested microorganisms was determined using crystal violet staining. For this purpose, new synthesized hydrogel beads containing 0.014 g of pure propolis were tested using release studies. Biofilm inhibition results were given at Table 3.

In respect of the obtained results, it was found that pure propolis samples had no inhibitory effect on biofilm formation especially Gram-negative test bacteria. Hydrogel beads were found to have a low antibiofilm activity. The antibiofilm activity of the carrier molecules was found to increase in a dose-dependent manner. The highest biofilm inhibition rates were observed on KE-2 hydrogel beads as 11.08% and 10.83% against *P. aeruginosa* and *S. typhimurium*, respectively. BM-2 hydrogel beads inhibited the biofilm formation of *S. typhimurium* at a percentage of 10.09%. In contrast to the antimicrobial activity results, hydrogel beads that did not contain CS were found to exhibit higher antibiofilm activities. Control group of hydrogel beads that did not contain propolis was also found to have antibiofilm activity.

4. Conclusions

In this study, antimicrobial and antibiofilm effects of PEs obtained from different regions of Mugla province on seven different test microorganisms were revealed. Subsequently, pH-sensitive GEL/SA and GEL/SA/CS hydrogel beads were synthesized in order to enable PEs to be transferred without degradation in the GI tract. The swelling rates of these hydrogel beads and the amount of propolis release in different pH buffer environments and simulated GI tract fluids were measured in a timed manner. In the study, antimicrobial and antibiofilm effects of newly synthesized pH-sensitive hydrogel beads were determined.

GEL/SA and GEL/SA/CS hydrogel beads showed the highest swelling rate in pH 6 buffer medium. In addition, the results of swelling ratio in simulated pH buffers and simulated GI tract buffers are similar in the carrier molecules.

Propolis release tests were carried out in different pH environments and simulated GI tract buffers of newly synthesized pH-sensitive PE-loaded GEL/SA and GEL/SA/CS hydrogel beads. The results of propolis release tests and swelling tests performed in different pH environments of hydrogel beads were found to be compatible. Accordingly, pH 6.8 was determined as the pH medium at which the maximum number of hydrogel beads were released. Among the newly synthesized GEL/SA/CS hydrogel beads in the pH 1.3 medium MIS-1, and in the pH 5, pH 6, and pH 6.8 medium KZ-3-encoded hydrogel beads exhibited the best propolis release. However, the KZ-5-encoded carrier molecule was found to possess the best propolis release from the GEL/SA hydrogel beads in the pH 1.3, pH 5, and pH 6 environments. It was found that the carrier molecules encoded as FE-2 had the best propolis release in pH 6.8 medium. In simulated GI tract environments (saliva, SGF, and SIF), MIS-1 from GEL/SA/CS hydrogel beads and MIS-2 from GEL/SA hydrogel beads were released maximum amount of propolis.

The results of antimicrobial activity of newly synthesized pH-sensitive hydrogel beads generally revealed that GEL/SA/CS carrier molecules exhibit higher antimicrobial activity against test microorganisms. In addition, there was no correlation between the propolis samples used as controls and the antimicrobial activity of the hydrogel beads. This is due to the fact that the propolis samples have different chemical structures as well as different binding reactions in the synthesized hydrogel beads.

For example, when the KZ-coded PE was loaded onto the hydrogel beads, it was found to have a higher level of antimicrobial effect on test microorganisms except for *Pseudomonas*. On the other hand, when KE-, MIS-, and FE-coded PEs were loaded on hydrogel beads, they showed lower antimicrobial activity to test strains except *C. albicans*. An increase in antimicrobial activities against some bacteria was observed when BM and MS-coded PEs were loaded especially

TABLE 3	Antibiofilm activity results of PE-loaded GEL/SA and GEL/SA/CS hydrogel beads						
M. organisms	B. subtilis *ATCC 6633*	S. aureus *ATCC 25923*	E. faecalis *ATCC 19433*	E. coli ATCC 25922	P. aeruginosa ATCC 27853	S. typhimurium ATCC 14028	C. albicans ATCC 10239
	% inhibition						
PE codes	Propolis extracts (0.014 g)						
KZ	6.25 ± 0.5	15.27 ± 0.8	10.01 ± 0.2	–	–	–	–
BM	4.6 ± 0.2	17.45 ± 0.9	4.53 ± 0.4	–	–	–	–
MS	6.72 ± 0.3	22.9 ± 1	0.97 ± 0.2	–	–	–	7.31 ± 0.8
KE	2.95 ± 0.1	5.55 ± 0.5	8.94 ± 0.5	–	–	–	–
FE	5.79 ± 0.2	13.49 ± 0.7	–	–	–	4.49 ± 0.6	–
MIS	–	7.5 ± 0.5	–	–	–	–	6.69 ± 0.1
Hydrogel codes	PE-loaded hydrogel beads						
KZ-3	5.42 ± 0.2	3.6 ± 0.2	2.11 ± 0.3	6.04 ± 0.5	5.38 ± 0.2	8.41 ± 0.5	4.53 ± 0.3
KZ-5	9.93 ± 0.3	9.59 ± 0.5	8.63 ± 0.5	4.56 ± 0.5	9.52 ± 0.5	5.36 ± 0.2	8.11 ± 0.4
BM-1	7.98 ± 0.3	6.36 ± 0.4	5.37 ± 0.4	7.95 ± 0.6	7.45 ± 0.4	8.94 ± 0.6	2.42 ± 0.2
BM-2	8.60 ± 0.5	7.30 ± 0.5	4.84 ± 0.6	8.59 ± 0.4	7.56 ± 0.5	10.09 ± 0.8	7.06 ± 0.5
MS-2	6.24 ± 0.1	2.82 ± 0.1	3.37 ± 0.1	0.95 ± 0.1	4.24 ± 0.2	1.05 ± 0.2	0.53 ± 0.1
MS-3	5.94 ± 0.3	4.90 ± 0.4	0.63 ± 0.1	3.92 ± 0.1	6.42 ± 0.4	6.94 ± 0.3	6.22 ± 0.4
KE-1	6.86 ± 0.5	5.42 ± 0.3	7.68 ± 0.5	0.74 ± 0.1	6.00 ± 0.5	5.36 ± 0.4	6.53 ± 0.5
KE-2	8.39 ± 0.5	8.86 ± 0.5	6.11 ± 0.3	9.76 ± 0.5	11.08 ± 0.7	10.83 ± 0.9	9.27 ± 0.5
FE-1	6.96 ± 0.6	5.21 ± 0.3	3.47 ± 0.2	6.79 ± 0.4	4.87 ± 0.4	6.96 ± 0.2	5.27 ± 0.3
FE-2	2.35 ± 0.1	0.73 ± 0.1	–	–	1.66 ± 0.1	2.57 ± 0.3	2.00 ± 0.1
MIS-1	2.25 ± 0.2	4.80 ± 0.5	4.00 ± 0.6	–	4.76 ± 0.1	4.73 ± 0.2	2.95 ± 0.2
MIS-2	–	–	–	–	–	1.79 ± 0.1	–
KL-1	4.71 ± 0.4	0.94 ± 0.1	2.95 ± 0.2	2.23 ± 0.2	4.24 ± 0.5	3.36 ± 0.3	0.84 ± 0.1
KL-2	6.96 ± 0.3	5.01 ± 0.5	2.95 ± 0.1	6.89 ± 0.7	6.11 ± 0.4	5.68 ± 0.5	6.85 ± 0.4

–, no inhibition.

in GEL/SA/CS hydrogel beads. Considering the propolis release test results, the increase in antimicrobial activity was found to be significant, especially in hydrogel beads coded KZ-3 and KZ-5.

The antibiofilm effects of PEs and PE-loaded hydrogel beads were detected to a low degree against all test strains. This is thought to be due to the continuation of biofilm production of test strains after the end of the propolis release from hydrogels.

In the study, synthesis of pH-sensitive hydrogel beads for different PEs was performed. These propolis-loaded hydrogel beads have been shown to increase antimicrobial activity in GI tract environments with different pH values and different time periods. GEL/SA/CS formula was found to be more effective in these hydrogel beads, though with PEs, release and antimicrobial effect differences were observed due to variation in chemical structure.

Another important conclusion is that these pH-sensitive hydrogel beads, which have propolis loaded on them, should first be checked for their effective pH values followed by biological activity tests after detection of the amount and duration of propolis release.

This study revealed that these pH-sensitive hydrogel beads synthesized for the transport of PEs without degradation for eliminating infections and cancer cells in each region of the GI tract are suitable carrier molecules provided that they are supported by *in vivo* cytotoxic experiments.

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6. Conflict of Interest

The authors declare have no conflict of interest.

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