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Antimicrobial Modified-Tragacanth Gum/Acrylic Acid Hydrogels for the Controlled Release of Quercetin

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Abstract

In this study, new antimicrobial hydrogels were prepared via reaction of functionalized-tragacanth gum (TG) biopolymer by quaternary ammonium functionalization of TG (QTG) with acrylic acid (AA). Characterization of the QTG hydrogels (QTG-AA) was carried out by FTIR, thermogravimetric analysis (TGA), and ^{1}H NMR. Dynamic mechanical analysis, (DMA) was conducted to characterize the mechanical behavior of hydrogels. Swelling behavior of the QTG hydrogels exhibited dependence on the pH, immersion time, medium, and temperature. Loading and *in-vitro* drug release from the final hydrogels were studied by using quercetin as a model drug. The results exhibited the pH-sensitivity of the hydrogels for drug delivery. The antimicrobial effect of the prepared hydrogels was investigated against some standard microorganisms and the results demonstrated that the hydrogels possess good antimicrobial activity against gram-positive and gram-negative bacteria as well as a fungus.

Keywords: *Release profile, Antibacterial, Drug release, Non-Fickian diffusion mechanism.*

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Introduction

Over the past years, outstanding researches have been made in the development of new biomaterials systems for improving drug delivery [1,2]. Drug delivery, especially site-specific delivery, is worthwhile for treatment of a various type of diseases and bacterial infections. For example, in combination with surgery, effective delivery of antimicrobial would be critical to prevent bone infection [3]. These stimuli hydrogels have ease of application because there is no need to surgery for the insertion of hydrogels into the body, therefore drugs, cells, and proteins can be mixed with the polymer solutions and simply administered by injection at specific and desired sites [4,5].

The utilization of natural and chemically modified polysaccharide hydrogels, as a part of drug development, has increased in the past two decades [6-8]. Great attention has also been focused on biopolymer based hydrogels for use as potential carriers in controlled drug delivery [9,10]. Natural polymer-based hydrogels have an advantage in comparison to the synthetic polymers due to their biodegradability, non-toxicity, eco-friendly low-cost production, and equilibrium swelling [11-14]. Tragacanth gum (TG), a high molecular weight carbohydrate biopolymer, is an exudate gum that founds in the desert of western and northern Iran. TG is one of the extensively used natural emulsifiers and thickeners available for drug, food, cosmetics, textile and adhesive due to its thermal stability, emulsifying ability, durability in a wide range of pH as well as high long shelf life [15-17]. Water-soluble part of TG is called tragacanthic acid or tragacanthin and the insoluble part called bassorin (~60%) which swell to a gel [18]. Tragacanthic acid is neutral consist of a complex mixture of bassorin and acidic branched hetero-polysaccharides which contain mainly D-galacturonic acid methyl ester [19,16]. Antimicrobial hydrogels have emerged as an essential platform to combat infections; they remain in place under physiological conditions while maintaining antimicrobial activity [20]. These attributes make them ideal for medical device, implant/catheter coatings, skin infections, and wound healing applications [21,22]. For instance, antimicrobial hydrogel of chitosan-dextran has been developed for endoscopic sinus surgery applications [23].

Quaternary ammonium compounds (QAC) are one of the most widely used biocides to inhibit microbial [24,25]. They are very effective at killing a broad spectrum of microorganisms such as gram-positive and gram-negative bacteria, yeast, and mold [26-30].

Therefore, the aim of this study was to synthesize new antimicrobial hydrogels based on the quaternary ammonium functionalization of TG (QTG). Then, it was polymerized with acrylic acid (AA) and monomers using *N*,*N*'-methylenebisacrylamide (MBA) as cross-linker. Swelling behavior of the prepared hydrogels and process of drug release from these hydrogels were studied with the variation of experimental conditions such as immersion time and medium pH. Various release

kinetics models were investigated to study the release profile of quercetin from the hydrogels. Finally, the antimicrobial activity of the materials was evaluated against *Escherichia coli*, *Pseudomonas aeruginosa* (Gram-negative) *Staphylococcus aureus*, *Bacillus subtilis*, (Gram-positive) bacteria anda fungus *Candida albicans* at neutral pH.

Experimental

Materials

The tragacanth gum (TG) with high quality was bought from a local pharmaceutical shop. Glycidyltrimethylammonium chloride (GTMAC), sodium hydroxide (NaOH), *N*,*N'*-Methylenebisacrylamide (MBA), Ceric ammonium nitrate (CAN), acrylic acid (AA), and quercetin were purchased from Sigma-Aldrich. Except for AA, which was distilled before use, all the chemicals were of analytical grade and used without further purification.

Synthesis of quaternary ammonium TG (QTG)

The purchased TG was sieved to remove dust and small particles, purified with ethanol extraction, dried under vacuum, ground to a fine powder and then kept in a desiccator before use. In a two-necked flask equipped with a magnetic stir bar, a nitrogen gas inlet tube, and a dropping funnel, TG (0.5 g) was dissolved in water (200 mL) and stirred for 24 h to achieve a homogenous mixture. Then, the pH of the solution was adjusted at 8 by addition NaOH solution (50% w/w) drop-wise under continues stirring. The mixture was stirred at 45 ˚C for 1 h, then 0.25 g GTMAC dissolved in 10 mL water was added to mixture and temperature was raised to 65 ˚C for 18 h. After neutralizing with nitric acid, the reaction mixture was poured into methanol to precipitate. Finally, the QTG product was filtered washed with acetone and then with ethanol and dried in vacuum oven at 40 ˚C.

Synthesis of QTG-AA hydrogels

in a 250 mL two-necked flask equipped with a magnetic stir bar and a nitrogen gas inlet tube, a certain weight of QTG (as listed inTable 1), was dispersed in bi-distilled water with stirring to achieve a homogenous mixture. The initiator solution (ceric ammonium nitrate dissolved in HNO_3, g/10 g HNO_3) was added to the mixture and stirring was continued at 50 ˚C for about 1 h. Then, a certain amount of AA monomer and MBA cross-linking agent (10 % based on the weight of AA) were dissolved in water and added to the reaction mixture and stirred for 6 h. A continuous supply of nitrogen was maintained throughout the reaction period. Finally, a few droplets of a 10%

aqueous NaOH solution were added to neutralize the mixture. The obtained hydrogel was dried in a vacuum oven at 40 ˚C till constant weight was reached.

Table 1. Features and compositions of the hydrogels.

Sample code	QTG (g)	AA (g)	MBA
QTG-AA	0.65	0.35	0.035
QTG-AA 50	0.5	0.5	0.05
QTG-AA 65	0.35	0.65	0.65

Characterization

FT-IR spectra were recorded in KBr pellets using a Bruker Tensor 27 spectrometer (Bruker, Germany). FT-IR spectra of all materials were collected in the 4000 cm^{-1} to 400 cm^{-1} region. ^{1}H-NMR spectra were recorded on a 400 MHz (Bruker Avance DRX, Karlsruhe, Germany) instrument in deuterium oxide (D_2O) by using tetramethylsilane as the internal standard. The integral values of ^{1}H NMR spectroscopy were used to evaluate the degree of quaternization (DQ) of TG with quaternary ammonium compound (QTG). DQ% was determined by the ratio of the integral area of methyl protons of quaternary ammonium compound(*N*,*N*,*N*-trimethyl) at δ 3.13 ppm (I_{NMe3}) to the integral area of the signal attributed to the methyl protons of the α-L-fucose unit at δ 1.10 ppm (I_{Me}), as shown in Eq. (1):

$$\mathrm{DQ}\ (\%) = 100 \times [\mathrm{I_{NMe_3}}/\mathrm{I_{Me}}] \times \frac{3}{9} \qquad (1)$$

Thermal stability of QTG-AA was examined using a thermogravimetric analysis (TGA Q50 V6.3 Build) under air atmosphere (flow of 20 ml min^{-1}) with the heating rate 10 ˚C/min from ambient temperature to 600 ˚C.

Gel fraction

Freshly prepared QTG-AA hydrogels were dried in an oven at 50 ˚C to a constant weight (W_0). The dried gels were extracted in a Soxhlet apparatus with hot distilled water for 24 h to remove the sol fraction. Then, the gels were dried to a constant weight (W_g). The gel fraction percent was calculated using the flowing equation:

$$\text{Gel fraction}\% = (W_g/W_0) \times 100 \qquad (2)$$

Swelling studies

The swelling behavior of hydrogels was studied in distilled water, in 0.9% NaCl solution, and in PBS. The effect of temperature, immersion time, and medium pH (2.2, 7.4 and 9.2) on the swelling ratio (SR) was investigated. To measure the SR, five samples of clean and dried QTG-AA were weighed and soaked in distilled water in the separate beakers. Similar experiments were also carried out for these hydrogel samples in 0.9% NaCl solution and in PBS at different pHs. The SR values of the samples were measured gravimetrically for different time intervals. To evaluate the swelling ratio at different pHs, the samples were dipped in PBS solutions (50 mL) at a predetermined pH ranged from 2.2 to 9.2. Furthermore, in the case of the effect of temperature, the samples were dipped in 50 mL solutions of PBS at pH 7 and left to remain for 8 h at different temperatures (15–45 °C) [31]. Before soaking, the dry weight (W_d) of each sample was determined. After soaking, the surface of samples was wiped with a tissue and then weighed (W_s). The swelling ratio was calculated from the equation 3. The average weight of five measurements was reported.

$$SR = \frac{(W_s - W_d)}{W_d} \qquad (3)$$

In vitro drug load

The quercetin solutions (1% w/v in PBS) were prepared at pH 7. A certain amount of dried samples of QTG-AA was immersed in separate beakers each containing 25 mL quercetin solution and incubated at 37 ˚C for 2 days. The samples were then taken out of the solutions and the remaining solution was diluted to 50 mL. Then, the amount of quercetin left in the solution was measured by UV-vis spectrophotometer (PG+90 Instrument, United Kingdom) at λ_{max}= 256 nm. The calibration curve standard at 256 nm was obtained by using diluted solutions of quercetin solution (1%, w/v). Entrapment efficiency (EE) of quercetin in the hydrogels was calculated by using equation (4)[32]:

$$EE = (\frac{W_i - W_f}{W_i}) \times 100 \qquad (4)$$

where $W_i$and $W_f$are the total amounts of quercetin in solution before and after loading, respectively.

In vitro drug release

To study the release behavior of quercetin from the samples, the quercetin-loaded hydrogels were placed in individual beakers each containing 100 mL distilled water, 0.9% NaCl solution, and PBS at pHs 2.2, 7.4 and 9.2. The beakers were incubated at 37±0.1˚C under constant shaking at 100 rpm.

From each beaker at predetermined time intervals, 4 mL of the release medium was taken out and replaced with 4 mL of fresh PBS to keep constant overall volume. The concentration of quercetin in the medium was determined by means of UV-vis spectrophotometer at 256 nm. All of the release experiments were performed in triplicate, and the averaged results were obtained.

Release kinetics data

Data achieved from the release studies were fitted to various release kinetic equations to comprehend the mechanism and kinetics of quercetin release from the samples. The quercetin released data were fitted to zero-order (eq. 5), first-order (eq. 6), Higuchi (eq. 7), Hixson–Crowell (eq. 8) and Korsmeyer–Peppas (eq. 9) models.

$$Q = Q_0 + k_0 t \quad (5)$$

$$\ln Q = \ln Q_0 + k_1 t \quad (6)$$

$$Q = k_H t^{0.5} \quad (7)$$

$$Q_0^{1/3} - Q_t^{1/3} = k_s t \quad (8)$$

$$Q/Q_\infty = k_k t^n \quad (9)$$

where Q and Q_∞ are the amounts of drug released at the time (t) and at equilibrium, respectively. Q_0 is the initial concentration of the drug in the solution. k_0, k_1, and k_H are the zero-order, first-order, and Higuchi release constant. For Hixson–Crowell equation (eq. 8) k_s is a constant incorporating the surface/volume ratio. For Korsmeyer–Peppas equation (eq. 9), k_k is the release rate constant that considers the geometric and structural features of the tablet, and n is the release or diffusional exponent which demonstrate the drug release mechanism. The value of n=0.5 demonstrates Fickian diffusion (Higuchi matrix), $0.5<n<1.0$ indicates anomalous (non-Fickian) diffusion, n=1.0 indicates case II transport (zero-order release) and $n>1.0$ indicates super case II transport [33,34].

Mechanical analysis

Dynamic mechanical analysis, (DMA) was conducted to characterize the mechanical behavior of hydrogels. The hydrogels (1 mm thick, 8 mm in diameter) were subjected to Zwick Roell Z050 (Germany) for compression cycles in the frequency range from 0.01 to 10 Hz at room temperature. The elastic modulus (E') and the viscous modulus (E") were measured as a function of frequency. The total number of discs per assay was 3.

Antimicrobial assay

The *in-vitro* antibacterial and antifungal activities of the samples were assayed using direct contact test with agar diffusion. For the preparation of tablets, about 100 mg of ground samples transferred to cylinder mold with a mobile shaft. Then under the force of 8 tons (Specac, USA) for 10 min, the tablets approximately with a thickness of 1 mm and 10 mm in diameter were fabricated. Then, the test compounds as tablets were placed on the surface of inoculated agar plates [35,36]. The antimicrobial activity of the samples was investigated against four bacterial and one fungal species. Test microorganisms consisted of *Candida albicans* PTCC 5027, *Escherichia coli* PTCC 1330, *Bacillus subtilis* PTCC 1023, *Pseudomonas aeruginosa* PTCC 1074, and *Staphylococcus aureus* ATCC 35923. All tested gram-negative and gram-positive bacteria were preserved as well as used for direct contact agar diffusion test in Muller-Hinton broth (Merck) except *C. albicans* that were cultured in SABOURAUD Dextrose broth (Merck). Before using the cultures, they were standardized with a final cell density of approximately 10^8 CFU mL^{-1}. The agar plates were inoculated from the standardized cultures of the test organisms by means of a sterile cotton swab and, then, it spread out as uniformly as possible throughout the entire media. The tablet was placed on the upper layer of the seeded agar plate. The SABOURAUD Dextrose agar plate and Muller-Hinton agar plate incubated at 37 °C for 24-48 hours. The antimicrobial activities of the hydrogels were compared with known antibiotic nystatin (100 Unit/disc), and gentamicin (10 µg/disc). Positive control plates were streaked with test microorganisms, but no tablet was used. Antimicrobial activity was evaluated by measuring the inhibition zone diameter (mm) on the surface of plates. Finally, the results were reported as mean ± SD after three repeats.

Results and discussion

Synthesis and characterization

Figure 1A shows FT-IR spectra of QTG and the QTG-AA hydrogel. The pristine TG showed absorption bands at 1745 and 1645 cm^{-1} attributed to stretching vibrations of C=O groups of ester and carboxylic acid, respectively, at 1100–1200 cm^{-1} related to vibration of C–O bond, and at 3445 and 2925 cm^{-1} due to O-H and C-H stretching, respectively [37]. Due to the broad fingerprint of absorption bands of TG in the range of 1800 to 600 cm^{-1}, some of the functional groups may have overlapped with each other. Therefore, it is hard to identify new functional groups' vibrations for QTG. QTG showed new absorption bands in the region of 1000 to 600 cm^{-1} (at 921, 717, and 686 cm^{-1}) which are due to stretching vibrations of the NR_4^+ complexes [36]. The absorption bands of C=O, N-H, and C-N overlapped with other absorption bands. The ^1H NMR technique was used to

characterize QTG and to determine the degree of substitution (DQ%). In 1H NMR spectrum of QTG, *Figure 1B*, the characteristic signal at δ 1.10 ppm corresponds to the methyl protons of α-L-fucose units, and the signal at 3.25 ppm is assigned to the vibrations of three pairs of protons of -[$N^+(CH_3)_3$] [38,39]. The solvent peak (D_2O) is assigned at δ 4.80 ppm. DQ% was determined by ^1H NMR and was about 41%.

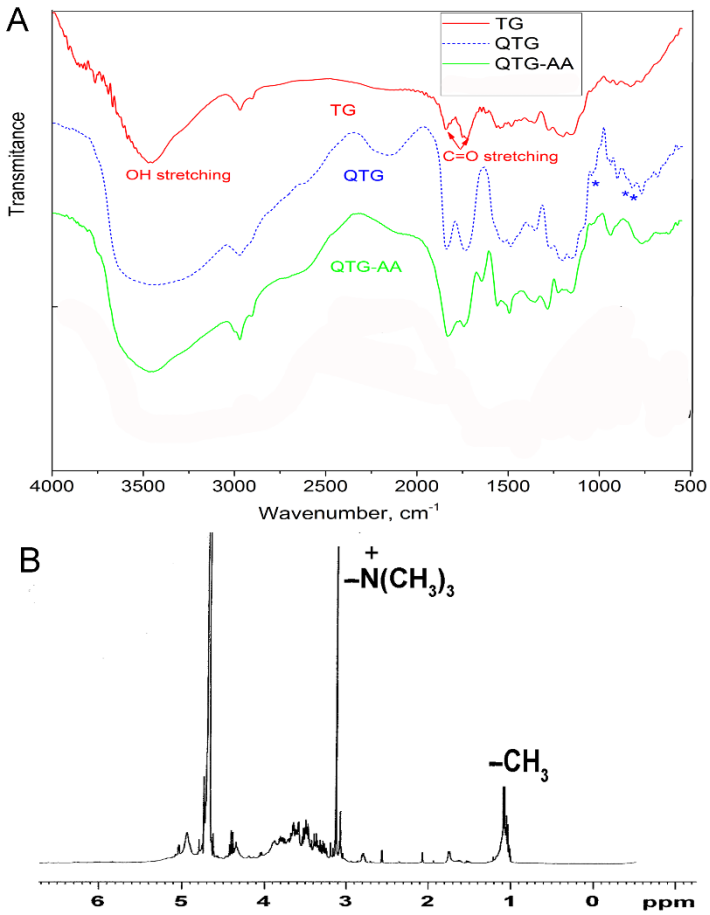

Figure 1. (A) FT-IR spectra of native TG, QTG, and QTG-AA hydrogel, (B) ^1H NMR spectrum for QTG.

Figure 2 shows TGA curves of modified TG and the corresponding hydrogels from 30 to 600 °C. For QTG, in the initial stage, there is dehydration and loss of volatile molecules which leads to the decomposition of modified TG, while in the lateral stage there is decomposition due to polymerization reaction [14]. In the TGA curves, the initial decomposition temperature and the char yield remained at 600 °C revealed that QTG-AA hydrogels were thermally more stable than modified tragacanth gum.

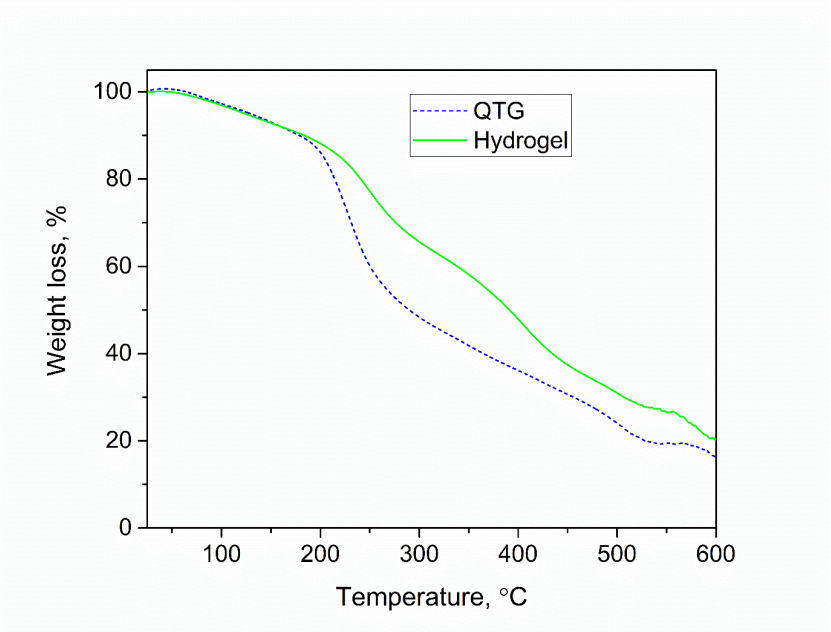

Figure 2. TGA curves of QTG and QTG-AA hydrogel.

Gel content and swelling

The gel content was obtained after 24 h hot extraction with distilled water. The gel fraction of QTG-AA was in the range of 60-62 wt%. The capacity of swelling is one of the most prominent factors that determine properties and applications of hydrogels.Swelling behavior is a function of many structural factors such as the charge, concentration, pK_{α} of ionizable groups, degree of ionization, cross-linking density, hydrophilicity and swelling medium such as temperature, ionic strength and counter ion [40]. Swelling behavior of the QTG-AA was studied in distilled water, in 0.9% NaCl solution, and in PBS at different pHs (2.2, 7.4, 9.2) and at different temperatures (25, 35, 45℃).Representative plots of the swelling ratios (SR) of the samples are presented in Figure 3A QTG-AA samples showed the highest and lowest SR values at pHs 9.2 and 2.2, respectively. This may be due to the opening of the pores caused by ionic repulsion of the constituted ions formed after partial hydrolysis of carboxylic acid groups at high pH values. Similar swelling trends have been observed in the case of swelling of polymers synthesized with other polysaccharides [41]. The water uptake by QTG-AA decreased in 0.9% NaCl solution which may be due to the screening effect of additional cations (Na^+) and decrease in the osmotic pressure difference between hydrogel network and external solution. It has been suggested that in saline solution, the mean pore size of the polymeric network of the hydrogels and molecular weight of polymer chain between two neighboring cross-links (Mc) decrease while crosslink density (ρ) and polymer volume fraction in the swollen state increase [34]. Dependence of swelling behavior of the hydrogels on temperature was determined at pH 7.4 for 24 h, and the results are shown in Figure 3B. Swelling of the hydrogels increased with increasing temperature of swelling medium. Other researchers have also studied the effect of temperature on the network structure of sodium alginate beads and observed an increase in Mc values with increasing temperature [42].

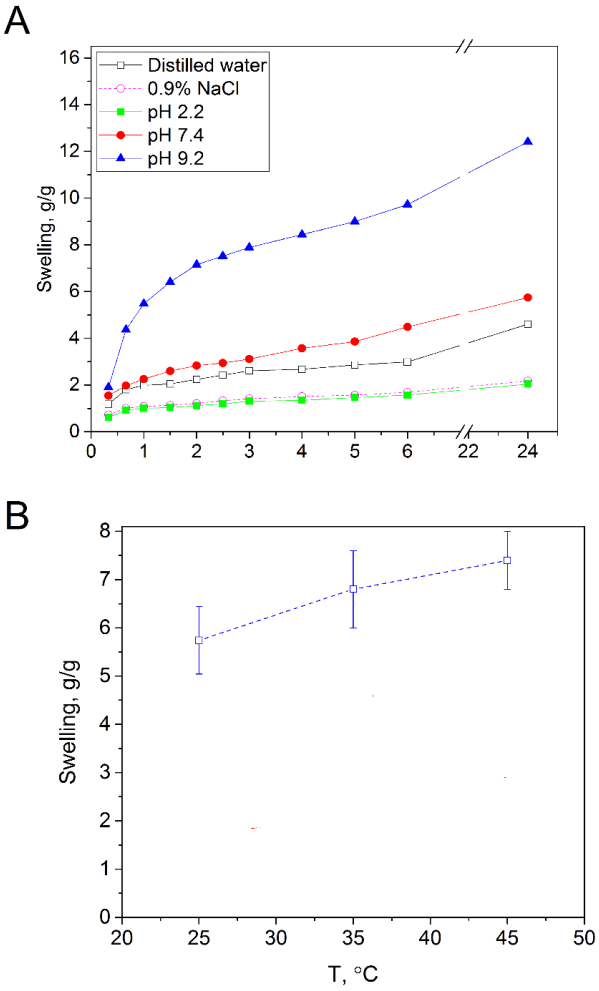

Figure 3. Effect of time on swelling ratio of QTG-AA hydrogels in different media (A) and effect of temperature at pH 7.4 (B). Each error bar represents one standard deviation and serves as the estimate of standard uncertainty.

In vitro quercetin load and release

The entrapped efficiency (EE%) of quercetin in the hydrogels was determined by UV-vis spectrophotometer and the results for the QTG-AA were very close to 65% and 61%, respectively. This similarity in the EE values is probably due to their polarity and interaction with quercetin. Quercetin release was investigated from drug loaded QTG-AA in PBS at three different pH values (2.2, 7.4, and 9.2). The release measurements were carried out during 24 h and the obtained results are shown in Figure 4. As can be seen, more release has been observed in pH 9.2 buffer for QTG-AA hydrogel as compared to the release in pHs 2.2 and 7.4. This can be due to higher swelling of QTG-AA hydrogel at pH 9.2, as shown in Figure 4 [43]. The network structure and pH sensitivity make these QTG based hydrogels candidate to be used as carriers for controlled drug release.

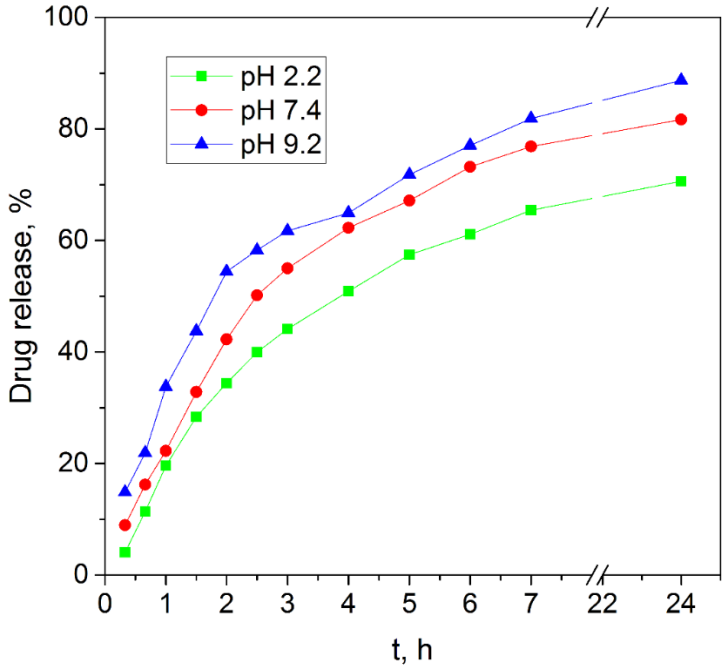

Figure 4. Effect of time on in vitro release profiles of quercetin-loaded QTG-AA hydrogel in PBS at different pHs.

Kinetic study of drug released

The release data of quercetin from the drug-loaded hydrogels were plotted according to the equations of relevant models and they are presented in Table 2. The release of drug from the loaded hydrogels depends on the swelling of the polymer matrix and solubility of the drug. Drug release from hydrophilic matrices has been shown to be a complex interaction between swelling, diffusion and erosion mechanisms [44]. In the present study, the higher swelling has been observed at pH 9.2 as compared to the swelling at pHs 2.2 and 7.4. Mathematical modeling of drug release can be very useful to speed up product development and to comprehend better the mechanisms controlling drug release from advanced delivery systems. According to the values of diffusion exponent '*n*', $0.5<n<1.0$, in Table 2, swelling of the hydrogels occurred through non-Fickian diffusion mechanism. In this mechanism, the rate of diffusion of water molecules into the hydrogels matrix and rate of polymer chains relaxation are comparable. On the other hand, the non-Fickian release mechanism implies that the release is controlled by diffusion or a combination of diffusion and macromolecular chain relaxation mechanisms. The results showed that the Higuchi and Korsmeyer–Peppas equation gave the best fit with the highest correlation coefficient (r^2) for all the hydrogels.

Table 2- Drug release parameters obtained by fitting release data by different release kinetics models.

samples	pH	Zero order		First order		Higuchi		Hixson-Crowell		Korsmeyer		
		k	r^2	k	r^2	k	r^2	k	r^2	n	k	r^2
QTG-AA	2.2	1.12	0.95	0.030	0.63	1.27	0.99	0.03	0.86	0.85	1.75	0.99
	7.4	1.25	0.95	0.026	0.70	1.67	0.97	0.29	0.83	0.71	1.91	0.97
	9.2	1.50	0.87	0.042	0.60	2.10	0.95	0.20	0.77	0.92	2.09	0.96

Mechanical properties

In Figure 5 the elastic and the viscous moduli curves as a function of the frequency of QTG-AA are reported. A chemical network is formed with AA presenting interconnections by covalent bonds with the MBA as cross-linking agent molecules. The storage modulus is higher than the loss modulus about more than one order of magnitude, which clearly reveals the elastic nature of these gels. Therefore, this hydrogel can be classified as a strong gel or a viscoelastic solid [45].

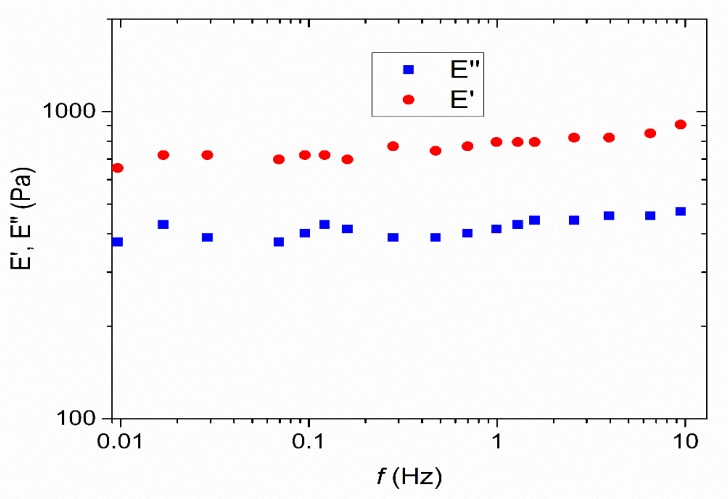

Figure 5. Mechanical properties of QTG-AA hydrogel. The plot of storage E' and loss E" shear moduli as a function of angular frequency. The tests were repeated at least three times on each sample.

Antimicrobial activity testing

Figure 6 shows the results of antimicrobial activity of samples. The unmodified-TG exhibited no inhibition zone while all other hydrogels containing quaternary ammonium showed antibacterial and antifungal activity. The finding towards inhibition of microorganisms was correlated with the standard antibiotics such as chloramphenicol and nystatin. All samples in this study, except TG, showed good activity against gram-negative and positive bacteria. In addition, antifungal activity revealed that the samples containing quaternary ammonium showed a good growth inhibitory effect against *C. albicans*. The antifungal activity of the tested samples was more effective than nystatin. Although the detailed mechanism for the antibacterial effect of quaternary ammonium salt has not

been determined, it is generally accepted that the mechanism of these materials is widely thought to be “contact killing”. A long, lipophilic alkyl chain penetrates bacterial cell membranes by binding to the cell wall components to produce leakage of the cytoplasmic material, autolysis, and cell death of bacteria [46,47].

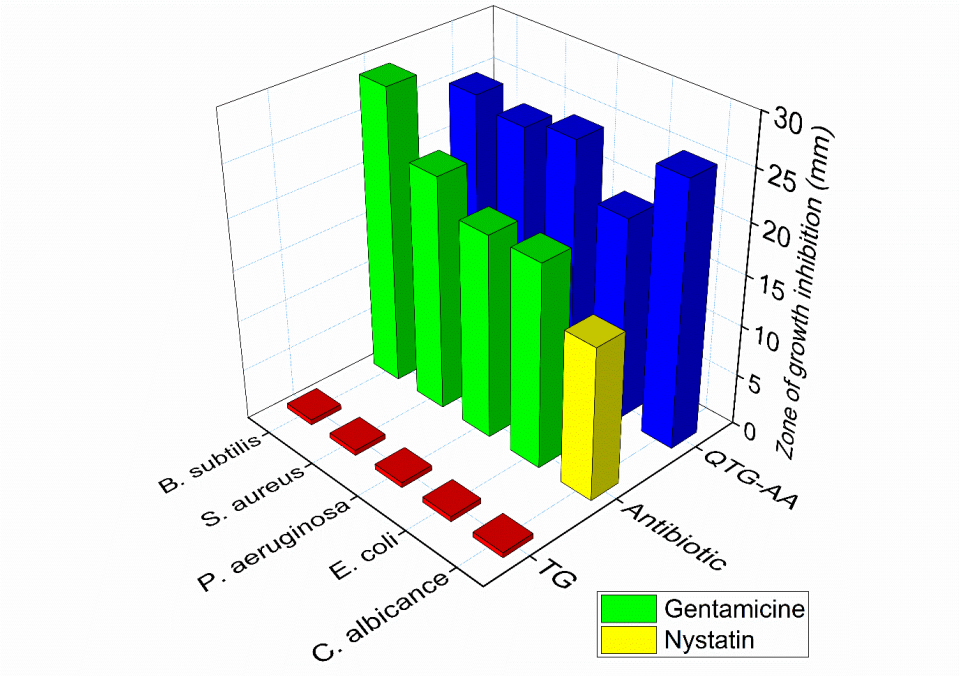

Figure 6. (A) The antimicrobial activity of samples against *E. coli*, *P. aeruginosa* (Gram-negative), *S. aureus*, *B. subtilis*, (Gram-positive) bacteria and a fungus *C. albicans*. The samples were compared with known antibiotics: nystatin, and chloramphenicol. *P <0.05 as compared to TG. Each error bar represents 1 standard deviation and serves as the estimate of standard uncertainty.

Conclusion

New natural based TG-AA hydrogel with antibacterial and antifungal activity was synthesized. Swelling medium affected the swelling ratio of TG-AA hydrogels. The hydrogels released quercetin in a controlled manner with salt- and pH-responsiveness properties. The quaternary ammonium grafted on TG (QTG), which has antimicrobial activity, can be immobilized in polymeric devices for biomedical applications including oral drug delivery systems.

Acknowledgments

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