Research paper

High-Payload chitosan microparticles for the colonic delivery of quercetin: Development and in-vivo evaluation in a rabbit colitis model

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ABSTRACT

Quercetin, a natural flavonoid has high potential for management of inflammatory bowel diseases (IBD). However, its onset of action is delayed when administered systemically and high doses are needed for IBD treatment. The present study aimed to develop chitosan microparticles for colonic delivery of clinically relevant quercetin concentrations as a potential treatment for IBD. Different formulations of quercetin microparticles were prepared and evaluated in terms of pharmaceutical, morphological and compatibility aspects. The in vitro release profiles of acid-resistant capsules filled with quercetin microparticles showed that most of quercetin was released in IBD colon simulating medium. Rabbit colitis model was used to evaluate the therapeutic effects of quercetin microparticles based on various assessment criteria (e.g. index of tissue edema, clinical activity score, colon macroscopic and histopathological characteristics, biochemical assays of the levels of myeloperoxidase enzyme and tumor necrosis factor-α and the activities of superoxide dismutase and catalase). The animals treated with quercetin microparticles had significantly improved therapeutic outcomes as compared to those treated with plain drug and the untreated animal controls. The results demonstrate that the developed quercetin microparticles have suitable pharmaceutical properties and might find clinical applications in acute IBD management.

1. Introduction

Inflammatory bowel diseases (IBD) are chronic autoimmune diseases affecting primarily the colonic mucosa. The factors causing IBD are unknown, but its incidence is influenced by various risk factors such as environmental pollution, microbial infections and genetic factors, which are the most essential independent risk factors. IBD has two principle types: Crohn's disease (CD) and ulcerative colitis (UC), which differ in genetic susceptibility, risk factors, and endoscopic, histological and clinical features. Although there is no sharp evidence on the relationship between IBD and mortality, IBD have undoubtful bad influence on patients' quality of life. Till date, there is no permanent cure for IBD. Chronic administration of drugs to minimize disease complications and ease patient's life is often necessary [1–4].

Quercetin (QUR) is one of the most abundant flavonoids, which are widely distributed in the main human dietary sources. QUR has anti-inflammatory and antioxidant actions. It was also shown to possess therapeutic effects in inflammatory conditions and aging and to reduce the risk of prostate, ovary, breast and colon cancers and leukemia [5–10]. The anti-inflammatory action of QUR is due to its inhibitory effect on inflammatory mediators (e.g. myeloperoxidase, lipoxygenase enzyme, interleukin 4 and tumor necrosis factor-α (TNF-α)) at site of inflammation. It also inhibits the recruitment of neutrophils and promotes the production of anti-inflammatory cytokines [10–13]. QUR has strong antioxidant properties due to its many phenolic hydroxyl groups that liberate hydrogen atoms to interact with the free radicals. Thus, QUR scavenges free radicals and/or chelates transition metal ions, terminates the radical chain, and modulates the endogenous antioxidants [14–17].

Owing to its antioxidant and anti-inflammatory actions, QUR...
showed promising protective and curative effects for the management of inflammatory bowel disease (IBD) [12,18]. The oral administration of QUR before induction of colitis in animal models has been proved to be highly protective against colitis complications [12]. However, oral administration of QUR after colitis induction had limited ameliorative effect. This is probably due to slow localization of the drug in pharmacologically sufficient concentrations at the site of inflammation after systemic absorption from the upper gastrointestinal tract [8,18].

Few colon-targeted delivery systems of QUR have been studied to achieve clinically relevant QUR concentrations in the colon, and thus enhance its therapeutic benefits in IBD [18–20]. All the developed systems had limited drug loading capacities ranging between 10% and 20%, which failed to achieve the relatively higher QUR doses needed in IBD (50–100 mg/kg/day) [12,18–20]. To date, there is only one study on the ameliorative effects of QUR-loaded microcapsules in an animal colitis model [18]. Accordingly, there is an unmet need for a delivery system that is able to deliver sufficiently high QUR concentrations to the colon.

Chitosan is a polysaccharide polymer synthesized by alkaline hydrolysis of chitin. It is composed of glucosamine and N-acetyl-D-glucosamine units linked through β-(1, 4) glycosidic linkages. The large number of amino groups present in chitosan structure is responsible for its pH-dependent solubility behavior; it is soluble in acidic pH but precipitates at pH higher than 7 [21]. This leads to premature drug release. The therapeutic potential of optimum QUR-loaded and unloaded microparticles. In a vacuum evaporator, the microparticles were mounted onto stubs, coated with gold.

Table 1

<table>
<thead>
<tr>
<th>Formulation</th>
<th>QUR (%)</th>
<th>Ferret’s diameter (mm) ± SD</th>
<th>Circularity ± SD</th>
<th>Roundness ± SD</th>
<th>Yield (%)</th>
<th>Drug loading (%) ± SD</th>
<th>Encapsulation efficiency (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.25</td>
<td>1.24 ± 0.08</td>
<td>0.97 ± 0.01</td>
<td>0.90 ± 0.05</td>
<td>33.8</td>
<td>24.9 ± 0.0</td>
<td>92.5 ± 0.2</td>
</tr>
<tr>
<td>F2</td>
<td>2.5</td>
<td>1.29 ± 0.08</td>
<td>0.97 ± 0.01</td>
<td>0.87 ± 0.06</td>
<td>43.0</td>
<td>36.5 ± 0.6</td>
<td>94.1 ± 1.7</td>
</tr>
<tr>
<td>F3</td>
<td>3.75</td>
<td>1.54 ± 0.09</td>
<td>0.97 ± 0.01</td>
<td>0.88 ± 0.06</td>
<td>47.0</td>
<td>49.1 ± 0.1</td>
<td>99.9 ± 0.2</td>
</tr>
<tr>
<td>F4</td>
<td>5</td>
<td>1.80 ± 0.10</td>
<td>0.96 ± 0.02</td>
<td>0.88 ± 0.05</td>
<td>52.3</td>
<td>53.3 ± 2.2</td>
<td>97.5 ± 4.0</td>
</tr>
<tr>
<td>F5</td>
<td>6.25</td>
<td>1.93 ± 0.11</td>
<td>0.95 ± 0.02</td>
<td>0.86 ± 0.05</td>
<td>53.3</td>
<td>60.7 ± 1.6</td>
<td>97.2 ± 2.6</td>
</tr>
<tr>
<td>F6</td>
<td>8.125</td>
<td>1.75 ± 0.13</td>
<td>0.96 ± 0.02</td>
<td>0.87 ± 0.05</td>
<td>57.4</td>
<td>65.3 ± 0.5</td>
<td>95.1 ± 0.8</td>
</tr>
<tr>
<td>F7</td>
<td>11.25</td>
<td>1.77 ± 0.13</td>
<td>0.95 ± 0.02</td>
<td>0.82 ± 0.08</td>
<td>62.0</td>
<td>72.3 ± 1.6</td>
<td>94.6 ± 2.0</td>
</tr>
<tr>
<td>F8</td>
<td>12.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*a* Microparticles were not formed.

2. Materials and methods

2.1. Materials

QUR and polyethyleneoxidecylether (Brij® 35) were purchased from Merck (Darmstadt, Germany). Sodium tripolyphosphate (TPP) and chitosan (Mw: 100,000–300,000 kDa, degree of deacetylation: 90%) were obtained from Acros Organics (Geel, Belgium). Sodium hydroxide and potassium dihydrogen phosphate were purchased from El-Nasr Pharmaceutical Chemicals Co. (Cairo, Egypt). Glacial acetic acid was purchased from SDFLC (Mumbai, India). Size three DRCaps™ acid-resistant capsules (made from hydroxypropyl methylcellulose) were supplied by Capsugel® (Bornem, Belgium). All other chemicals were provided as analytical grades.

2.2. Microparticles preparation

Ionic cross-linking technique was followed to prepare the microparticles [24]. For chitosan solution (2.5%, w/v) preparation, chitosan was dissolved in acetic buffer at pH 5 and the solution was magnetically stirred for 1 h. To the resultant chitosan solution, QUR was added and uniform dispersion was prepared by magnetic stirring for 30 min. The resultant QUR dispersion (2 g) was dropped through a needle (400 μm inner diameter) into 10 mL TPP solution (2%, w/v) under continuous stirring for 15 min at 200 rpm. The microparticles were collected by sieving then washed in triplicate with distilled water and allowed to dry for 24 h at ambient temperature. Different concentrations of QUR were investigated to select the optimum microparticles composition ratio (Table 1). The same procedures were utilized to prepare unloaded chitosan microparticles and used as a control.

2.3. Microparticles characterisation

2.3.1. Size and morphology

A sample of 20 microparticles of each formula were mounted on a calibrated stage and digital photographs were captured using Canon® digital camera SX 230 HS (Tokyo, Japan). A computerized image analysis software (ImageJ®, Maryland, USA) was used to calculate the average Feret’s diameter and sphericity of the microparticles [27]. Roundness and circularity were calculated based on equations (1) and (2), respectively.

\[
\text{Circularity} = 4\pi \times \frac{(\text{area})}{(\text{perimeter})^2} \tag{1}
\]

\[
\text{Roundness} = \frac{4 \times (\text{area})}{\pi \times (\text{major axis})^2} \tag{2}
\]

Scanning electron microscope (Jeol, JSM-5200, Tokyo, Japan) at low energy (15 kV) was used to study the morphological features of the optimal QUR-loaded and unloaded microparticles. In a vacuum evaporator, the microparticles were mounted onto stubs, coated with gold.
and photographed at different magnifications (15, 50 and 2000×).

2.3.2. Yield, drug loading capacity and drug encapsulation efficiency

The collected QUR microparticles were weighed and the yield of each batch was calculated using equation (3). For drug content determination, QUR microparticles were grinded and a specific weight was dissolved into 100 mL 0.1 N HCl solution containing Brij® 35 (0.5%) by continuous stirring for 24 h. Drug solution was filtered and drug concentration was determined by a UV–Vis spectrophotometer (Genway, England) at wavelength of 255 nm. The Drug loading capacity (DL) and the encapsulation efficiency (EE) were calculated using equations (4) and (5), respectively.

\[
\text{Yield (\%)} = \frac{\text{Weight of collected microparticles}}{\text{Theoretical weight of microparticles}} \times 100
\]

\[
\text{DL (\%)} = \frac{\text{Weight of drug in microparticles}}{\text{Weight of microparticles}} \times 100
\]

\[
\text{EE (\%)} = \frac{\text{Weight of drug in microparticles}}{\text{Initial drug weight}} \times 100
\]

2.3.3. In vitro drug release studies

The release of QUR from acid-resistant capsules loaded with the selected microparticles (equivalent to 7 mg QUR) was examined in comparison to that from the capsules loaded with the plain drug (7 mg). The capsules were inclosed in stainless steel baskets suspended in tightly-closed vessels containing 100 mL of different release media. The vessels were shaken at 50 rpm in a temperature-controlled shaking water bath (37.0 ± 0.5 °C). Drug release was examined firstly for 2 h in simulated gastric medium (i.e. 0.1 N HCl solution, pH 1.2), then in simulated intestinal medium (i.e. phosphate buffer, pH 6.8) for 4 h, and then followed by additional 24 h in IBD colon simulating medium (i.e. acetate buffer, pH 4.0) [25, 28] or healthy colon simulating medium (i.e. phosphate buffer, pH 7.2). All the media contained Brij® 35 (0.5%). Aliquots (3 mL) were taken from the vessels at specified time intervals and filtered. The withdrawn samples were replenished directly by 3 mL of fresh release medium. The amounts of drug released were determined by spectrophotometric analysis at wavelength of 255 nm.

2.3.4. Differential scanning calorimetry (DSC) studies

DSC analyses of chitosan, unloaded microparticles, QUR and QUR microparticles were carried out using a computer-interfaced calorimeter (Shimadzu, Model DSC-50, Kyoto, Japan). Each sample (3–5 mg) was put in an aluminum pan, closed and heated at a rate of 10 °C/min in the range of 25–350 °C under constant nitrogen gas flow.

2.3.5. Fourier transform-infrared spectroscopy (FT-IR) studies

FT-IR analyses of chitosan, unloaded microparticles, QUR and QUR microparticles were carried out using JASCO FT-IR–4200 type A (Tokyo, Japan). Each sample (3–4 mg) was mixed with potassium bromide (IR grade). The mixtures were compressed to form disks. The scanning was carried out in the range of 4000–400 cm⁻¹.

2.4. In vivo evaluation

2.4.1. Experimental animals

Twenty-two adult male New Zealand white rabbits (Total Company, Assiut, Egypt) of 1.8 kg average weight were housed in individual cages at controlled temperature (25–27 °C) and 12-h light/dark cycle. The rabbits were allowed to adapt to the housing conditions for 3 days before experimentation with free access to food and water. The animals were randomly divided into 4 groups: normal untreated group (n = 5), colitis untreated group (n = 7), QUR group (n = 5) and QUR microparticles group (n = 5). Animal experiments were approved by the Institutional Animal Ethical Committee of the Faculty of Pharmacy, Assiut University and it adhered to the Guide for the Care and Use of Laboratory Animals, 8th Edition, National Academies Press, Washington, DC.

2.4.2. Colitis induction

All rabbits were fasted with free access to water 24 h before the experiments. A lubricated soft infant feeding tube was inserted into the anus of rabbits for 20 cm. Using a plastic syringe fitted onto the feeding tube, 1.5 mL acetic acid solution in saline (4%) was administered slowly. Then, an empty plastic syringe was fitted onto the tube and 7 mL air was pushed rapidly to spread the administered solution throughout the colon. The animals were kept in a head-down position for 30 s to inhibit the leakage of the solution. Then, 7 mL saline was instilled through the feeding tube to flush the colon and the animals were kept in the same position for additional 15 s. Finally, the rabbits' position was reversed to allow the withdrawal of excess fluids. Rabbits were returned back to cages and access to water and food ad libitum. In the case of the normal group, the same procedures were followed, except of instilling saline instead of the 4% acetic acid solution [29, 30].

2.4.3. Treatment protocol and sample collection

The rabbits of the colitis group and the normal group did not receive any treatment. One acid-resistant capsule loaded with QUR (an amount equivalent to a dose of 50 mg/kg of rabbit weight) was administered for each of the QUR-treated rabbits every day while the QUR microparticles treated rabbits were administered one acid-resistant capsule filled with QUR microparticles (equivalent to QUR dose of 50 mg/kg) every day. The treatment started 1-h after colitis induction and lasted for 3 consecutive days in both groups. One day after the last dose, all the rabbits were sacrificed by cervical decapitation and the distal colons were removed directly. Each colon was incised longitudinally and the stool bleeding and stool consistency were scored. After that, each colon was swilled with saline to remove the feces for macroscopic scoring. The inflamed piece of the colon was cut into two pieces. The first piece was frozen immediately using liquid nitrogen and then stored at -20 °C for biochemical assays. The other piece was stored in formalin solution (10%) for microscopic examination [30].

2.4.4. Treatment assessment

2.4.4.1. Colitis severity assessment. The clinical activity score system was calculated by scoring each of stool consistency, stool bleeding and weight loss, as reported by Hartmann et al. with minor modulations (Table 2) [31]. The summation of these scores ranged from 3 (healthy to 12 (severe colitis). A segment (25-cm) of colon that had major gross pathologic changes was incised and weighed. The index of tissue edema was calculated as the ratio between the wet weight of the colon segment and the body weight [32].

2.4.4.2. Macroscopic assessment of the colonic damage. The grading scale of Morris et al. (Table 3) was used to assess the macroscopic damage of the colonic mucosa [33].

2.4.4.3. Histological evaluation of the colonic damage. The pieces of colon tissues fixed in formalin were trimmed, dehydrated and

Table 2

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight loss</th>
<th>Stool consistency</th>
<th>Stool bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Weight gain &gt; 1%</td>
<td>Well-formed pellets</td>
<td>Normal color stool</td>
</tr>
<tr>
<td>2</td>
<td>Weight loss or weight gain &lt; 1%</td>
<td>Semi-formed pellets</td>
<td>Brown color stool</td>
</tr>
<tr>
<td>3</td>
<td>Weight loss between 1 and 5%</td>
<td>Pasty stool</td>
<td>Reddish color stool</td>
</tr>
<tr>
<td>4</td>
<td>Weight loss &gt; 5%</td>
<td>Watery stool</td>
<td>Bloody stool</td>
</tr>
</tbody>
</table>
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The tube was incubated again for 5 min. Finally, 100 μL of 5-thio-2-nitrobenzoic acid (TNB) solution (or 100 μL of distilled water for standard preparation) was added to stop the reaction and the color turned yellow immediately. The activity of myeloperoxidase (MPO) enzyme was determined by colorimetry using NWLSS® Myeloperoxidase Activity Assay Kit (AMS Biotechnology, UK) [34,35]. In a microcentrifuge tube, 880 μL of phosphate-buffered saline pH 6.5 (containing β-amino taurine) and 80 μL sample solution (or 80 μL assay buffer to Zero MPO Standard) were placed and the tube was incubated in a water bath (25 °C) for 5 min. Then, 40 μL of 100 μM H2O2 solution was mixed quickly and the tube was incubated for 30 min. MPO catalyzed the reaction between hydrogen peroxide and chloride forming hypochlorous acid (HOCl) which was rapidly trapped by taurine forming oxidant taurine chloramine and hydrogen peroxide and chloride forming hypochlorous acid (HOCl) which was rapidly trapped by taurine forming oxidant taurine chloramine (TauNHCl) which is stable. Afterward, 40 μL of catalase (CAT) solution was added to eliminate hydrogen peroxide and to stop the MPO catalyzed reaction. The tube was incubated again for 5 min. Finally, 100 μL of 5-thio-2-nitrobenzoic acid (TNB) solution (or 100 μL of assay buffer for blank) was added and mixed well and the tube was incubated again for 20 min. The absorbance of the samples was measured at 412 nm (Beckman DU 7400, USA) and the units of MPO activity in sample solution were calculated as reported.

2.4.4.4. Biochemical assays. One gram of each colon piece was homogenized into 10 mL phosphate-buffered saline (50 mM, pH 7.0). Then, the samples were centrifuged at 10,000 RPM at 4 °C for 30 min. Finally, the supernatants were taken for analysis.

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TNF-α concentration in the samples was measured using rabbit TNF-α Mybiosource® ELISA kit (San Diego, USA). Aliquot (100 μL) of sample solution was added and mixed gently into a well of microplate coated with a monoclonal antibody specific for rabbit TNF-α. Into another well, 100 μL distilled water was used instead of sample solution to prepare a control. Samples (100 μL) of TNF-α standard solutions having different concentrations (15.6, 31.3, 62.5, 125, 250, 500 and 1000 pg/mL) were treated similarly and standard curve was constructed. After filling the wells, the microplate was incubated at room temperature for 90 min. Thereafter, 100 μL of biotinylated detection antibody working solution was added to the wells and the microplates were incubated again at room temperature for 1 h. Unbound antibodies were removed by aspirating each well and washing with 350 μL washing buffer for three times. Then, 100 μL avidin-horseradish peroxidase conjugate solution was added and the microplate was incubated for 30 min at room temperature. The aspiration/wash cycles were repeated for five times. After that, 90 μL of substrate solution (color reagent) was added to allow the reaction between the enzyme and the substrate yielding a blue product. After incubation for 15 min at room temperature and away from light, 50 μL of stop solution was added to stop the reaction and the color turned yellow immediately. Within 30 min, the optical densities of the wells were determined using a microplate reader (Stat Fax 2300, USA) at 450 nm and TNF-α concentrations in the samples were calculated.

### Table 3

<table>
<thead>
<tr>
<th>Score</th>
<th>Macroscopic features</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Intact undamaged tissues</td>
</tr>
<tr>
<td>1</td>
<td>Localized hyperemia without ulceration</td>
</tr>
<tr>
<td>2</td>
<td>Single ulceration site without inflammation</td>
</tr>
<tr>
<td>3</td>
<td>Single ulceration site with inflammation</td>
</tr>
<tr>
<td>4</td>
<td>Two or more ulceration sites with inflammation and the total ulcers size &lt; 1 cm</td>
</tr>
<tr>
<td>5</td>
<td>Two or more ulceration sites with inflammation or one major ulceration site with inflammation extending &gt; 1 cm along the colon length</td>
</tr>
</tbody>
</table>

embedded in paraffin blocks. The blocks were sectioned into 5-μm thickness sections and stained with acridine orange (AO) stain, periodic acid Schiff and hematoxylin (PAS&H) stain and hematoxylin and eosin (H&E) stain. The colonic damage of the samples was evaluated by light microscope (Olympus BX51, Japan) and the photos were captured by a digital camera (Olympus DP72, Japan) fitted to the microscope.

### 2.5. Statistical analysis

GraphPad Prism® version 5.00 for Windows (San Diego, California, USA) was used to perform all statistical analyses. All experimental data were expressed as the mean ± standard deviation of at least three independent measurements. One-way analysis of variance (ANOVA) with Tukey post-hoc test was performed for multiple comparisons between formulations with respect to pharmaceutical and morphological features. One-way ANOVA with Dunnett post-hoc test was performed to compare between the different groups of animals and the normal group in terms of the treatment assessment. To compare the different animal groups, Tukey post-hoc test was performed. Statistical significance was set at p < 0.05.

### 3. Results and discussion

#### 3.1. Microparticle preparation and optimization

Microparticles were created via ionotropic cross-linking of chitosan with TPP [39,40]. Different formulations of QUR microparticles (F1–F8) were prepared and evaluated based on yield, EE, DL and morphological characters to select the optimal microparticles. The concentrations of chitosan and TPP were previously optimized to prepare spherical microparticles of dense cross-linked matrix that have limited swelling and drug release in the simulated intestinal medium [24].

QUR microparticles were prepared at different QUR/chitosan weight ratios (0.5:1–5:1). Increasing QUR/chitosan ratio from 0.5:1 to 4.5:1 (F1–F7) did not disrupt the formation of intact microparticles, while no microparticles were formed at the highest QUR/chitosan ratio 5:1 (F8), presumably due to the high amount of drug in the droplet matrix, which might interrupt the instant cross-linking process of chitosan by TPP (Table 1).

The microparticles prepared at lower QUR/chitosan ratios (F1–F3) had small sizes (1.24 ± 0.08 to 1.54 ± 0.09 mm, Table 1). By increasing QUR/chitosan ratio (F4–F7), the microparticles showed larger...
sizes, likely because of the increased drug content in the microparticles. The sphericity of the microparticles prepared at different QUR/chitosan ratios was almost similar, except a slight reduction in the roundness of the microparticles formed at the ratio of 4.5:1 (F7), probably because of the increased drug loading within the microparticles (Table 1).

Generally, all formulations had high and similar EE due to the poor aqueous solubility of QUR [41] and the optimal chitosan and cross-linker concentrations used. Therefore, the diffusion of drug from the microparticles during cross-linking and washing processes was limited [42]. By increasing QUR/chitosan ratio, the yield and DL of the prepared microparticles increased due to increased drug content in the microparticles. Maximum yield and DL were observed with the microparticles prepared at the highest QUR/chitosan ratio of 4.5:1 (F7) (Table 1).

3.2. Characterizations of the optimal formulation

3.2.1. SEM studies
The unloaded microparticles images show small sized (1.23 ± 0.12 mm) and almost spherical microparticles with dense and smooth surface (Fig. 1a and b). In contrast, the QUR microparticles were larger in size (1.77 ± 0.13 mm) and slightly less spherical (Fig. 1c and d). The size and sphericity differences between unloaded and loaded microparticles might be due to the entrapment of QUR that probably have partially disrupted the network structure of the cross-linked chitosan. Images at higher magnification (Fig. 1d) show cracked rough surface with the drug particles embedded into the cross-linked matrix.

3.2.2. DSC studies
Chitosan thermogram shows an endothermic peak at 124.2 °C characteristic for evaporation of surface-bound water and another exothermic decomposition peak at 305.6 °C (Fig. 2a) [44]. The unloaded microparticles thermogram shows the same water evaporation peak at 121.2 °C, but more intense because the cross-linked chitosan has higher water holding capacity than the non-cross-linked one. The second peak of chitosan decomposition appeared at lower temperature (243 °C) (Fig. 2b). The presence of unsubstituted amine groups in the non cross-linked chitosan might have consumed higher energy for degradation [44,45]. QUR thermogram shows an endothermic peak at 138.3 °C due to the release of water molecules (i.e. water of crystallization) which are strongly held by crystal lattice, thus requiring higher temperature. The thermogram shows a second endothermic peak of QUR melting at 327.3 °C and a third exothermic peak of QUR decomposition at 363.9 °C (Fig. 2c) [41,46]. The thermogram of the QUR microparticles shows an endothermic peak of water evaporation at 136.7 °C followed by an exothermic peak of chitosan decomposition at 292.4 °C. The thermogram also shows an endothermic peak at 307.1 °C, which might be due to the melting of QUR. The downward shift of this peak, as well as decreased intensity as compared to the original QUR peak might be due drug dispersion in the microparticle matrix (Fig. 2d) [47].

3.2.3. Fourier transform-infrared spectroscopy studies
Chitosan spectrum shows a broad vibration band at 3000–3700 cm⁻¹ of OH groups stretching overlapped with the stretching vibration band of NH groups [48]. The spectrum also shows a
distinctive stretching vibration band of amidic C=O group at 1651 cm$^{-1}$ and a bending vibration band of amidic NH group at 1595 cm$^{-1}$ (Fig. 3a) [27]. The unloaded microparticles spectrum shows the same OH groups stretching band (3000–3700 cm$^{-1}$). However, the cross-linking of chitosan resulted in shifting both amidic C=O and NH groups bands to 1649 cm$^{-1}$ and 1556 cm$^{-1}$, respectively [49,50]. Moreover, a characteristic band of P=O (i.e. corresponding to TPP) appeared at 1092 cm$^{-1}$ (Fig. 3b) [44]. In the spectrum of QUR, the stretching vibration of O–H groups is observed as a broad band at 3035–3581 cm$^{-1}$ due to the presence of water molecules. The spectrum also shows the characteristic stretching vibration band of aromatic ketonic C=O at 1664 cm$^{-1}$, the stretching vibration band of C=C at 1560 cm$^{-1}$ and the stretching vibration band of C–OH at 1381 cm$^{-1}$ (Fig. 3c) [51,52]. The QUR microparticles spectrum shows the characteristic bands of the drug and the cross-linked chitosan, almost at the same wave numbers revealing the absence of chemical interactions between the microparticles components and the drug (Fig. 3d).

3.2.4. In vitro release study of QUR microparticles filled capsules

All release media contained the nonionic surfactant Brij$^\text{®}$ 35 (0.5%, w/v) to enhance the dissolution of the poorly soluble QUR and maintain sink conditions [19]. As the capsules are acid-resistant, they remained
intact in simulated gastric medium for 90 min [53]. By the end of 2 h, the capsules swelled slightly and ca. 30.3% of QUR was released from the capsules filled with plain drug, whereas only ca. 4.8 ± 0.3% of the drug was released from the capsules filled with drug-loaded microparticles (Fig. 4). In simulated small intestinal fluid (phosphate buffer, pH 6.8) the capsules were ruptured and most of the plain drug ca. 90.8% was released in the medium after 1 h, while the drug was released slowly from chitosan microparticles due to the limited chitosan swelling at this pH [40]. By the end of 6 h (pH 1.2 and pH 6.8), the entire plain drug was released, while the release of QUR from the microparticles was ca. 18.1 ± 1.3%.

In the medium simulating healthy colonic pH of 7.2, the cumulative released percentage of drug from the chitosan microparticles after 30 h was 75.7 ± 5.8%. On the contrary, in the medium simulating IBD colon (acetate buffer, pH 4.0), the release rate increased sharply and most of the loaded drug ca. 93.6 ± 2.65% was released after 10 h, probably due to extensive chitosan swelling in this medium (Fig. 4). These results confirm the ability of the developed system to selectively deliver the loaded drug under conditions simulating the IBD colon, which could enhance drug efficacy and reduce the required dose.

3.3. Treatment assessment

Colitis results in intensive damage of colonic mucosa and heavy infiltration of inflammatory cells. Rectal bleeding, watery diarrhea and body weight loss are characteristics for colitis [29,54,55]. The developed rabbit colitis model was confirmed by the assessment of several pathological and biochemical parameters. The therapeutic efficacy of the QUR microparticles vs. the plain drug was evaluated.

3.3.1. Colitis severity assessment

Both the colitis group and the QUR group had clinical activity scores significantly higher than that of the normal group (p < 0.001). In contrast, the clinical activity score of QUR microparticles group did not differ significantly from the score of the normal group (p > 0.05) (Table 4).

The colitis group and the QUR group had indices of tissue edema significantly higher (p < 0.05 and p < 0.001, respectively) than that of the normal group. The index of tissue edema of the QUR microparticles group was comparable to that of the normal group (Table 4). Based on these results it is reasonable to conclude that the therapeutic effect of QUR microparticles is better than that of the plain drug.

3.3.2. Macroscopic assessment of the colonic damage

Macroscopically, the normal group colons were intact, while the colons of both the colitis group and the QUR group were inflamed intensively and had numerous wide areas of ulceration. The macroscopic scores of the colitis group and the QUR group were significantly higher than that of the normal group (p < 0.001). Generally, hyperemia, sometimes with small areas of ulceration and inflammation, was shown in the colons of the QUR microparticles group rabbits (i.e. slightly higher non-significant macroscopic score than that of the normal group, p > 0.05) (Table 4).

3.3.3. Histological evaluation

The histopathological examination of the normal group colons showed normal histology and architecture. Normally, the lamina epithelialis formed of simple columnar epithelium with PAS-positive goblet cells. Apoptotic cells were observed in a few numbers. The submucosa was normal and formed of loose connective tissue containing crypts of lieberkühn (intestinal glands) and free from leukocyte infiltration (Fig. 5a1–5a3) [56].

Intensive mucosal injury was observed in the colitis group colons. Erosion of surface epithelium, ulcerative colitis, cryptitis and crypt abscesses were observed intensively. The intestinal glands were degenerated and the muscularis mucosae was thickened and degenerated [56,57]. Depletion and degeneration of the PAS-positive goblet cells were also recognized. Apoptotic cells stained with AO were found in large numbers, reflecting oxidative damage of DNA [58]. The submucosa was observed with leukocyte infiltration and edema, because of the increased vascular permeability and the release of inflammatory mediators (Fig. 5b1–5b3).

Moderate to severe mucosal injury was exhibited in the colons of the QUR group. Ulcerative colitis, cryptitis, crypt abscesses and intestinal glands degeneration were recognized distinctly. The PAS-positive goblet cells were depleted and degenerated obviously. Increased apoptotic cells number was observed. Thickening and degeneration of the muscularis mucosae were also recognized. Furthermore, submucosal leukocyte infiltration was detected moderately (Fig. 5c1–5c3).

Generally, the colons of the QUR microparticles-treated rabbits showed almost normal histological architecture. Normal distribution of the PAS-positive goblet cells in the lamina epithelialis and within the crypts of lieberkühn was recognized and the cells reached normally to the muscularis mucosa. Limited number of apoptotic cells was detected. The submucosa was thin and limited edema and leukocyte infiltration were observed (Fig. 5d1–5d3).

3.3.4. Biochemical assays

3.3.4.1. Anti-inflammatory activity assessment. In IBD, the inflammatory enzyme MPO is responsible for the production of hypochlorous acid that impairs the stability of colonic cell membrane and causes cell death by lipid peroxidation [59,60]. TNF-α is an inflammatory mediator that is overproduced from leukocytes in case of IBD. TNF-α leads the inflammation reactions cascade producing peroxide anions and nitrogen/oxygen radicals that cause damage of colon tissues [55].

In the colitis group, the MPO enzyme activity and the TNF-α concentration were significantly higher (p < 0.001) than the normal group. This indicates the incidence of acute inflammation and confirms the histologically observed leukocyte infiltration (Table 4).

The levels of MPO and TNF-α in the QUR group were significantly higher (p < 0.05 and p < 0.01, respectively) than those of the normal group. However, TNF-α level was significantly lower (p < 0.05) than that in the colitis group, whereas the level of the MPO did not significantly differ from that of the colitis group (p > 0.05) (Table 4). This result is supported by the histologically observed moderate submucosal leukocyte infiltration in this group of animals.

The level of MPO in the QUR microparticles group was comparable to that of the normal group. Although the level of TNF-α was significantly higher (p < 0.05) than that in the normal group, it was decreased significantly as compared to its level in the colitis group (p < 0.01) (Table 4).
Antioxidant activity assessment. As antioxidant enzymes, CAT and SOD have a major defensive role against reactive oxygen species, and thus prohibit the pathological changes in the cellular proteins, lipids and DNA. In IBD, the oxidative stress in the colon results in considerable consumption of those antioxidant enzymes [60,61]. CAT and SOD activities in the colons of the colitis group rabbits were significantly lower (p < 0.01 and p < 0.001, respectively) than the activities in the normal group (Table 4).

The activities of CAT and SOD enzymes in the QUR group were significantly lower (p < 0.05) than the enzymes activities in the normal group. As compared to the colitis groups, CAT activity was not significantly different (p > 0.05), while SOD activity was significantly higher (p < 0.05) (Table 4). The minimal anti-inflammatory action and the limited restoration of the antioxidant enzymes activities indicate the limited localization of plain QUR in the colon as a result of its absorption from the upper gastrointestinal tract [8,62,63]. Previous studies showed limited curative activity of the orally administered plain drug on induced colitis [18].

In the QUR microparticles group, the activities of CAT and SOD were restored almost completely, and thus their activities in this group

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Normal</th>
<th>Colitis</th>
<th>QUR</th>
<th>QUR microparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical activity score</strong></td>
<td>3.0 ± 0.0</td>
<td>8.4 ± 1.7**</td>
<td>10.0 ± 0.0***</td>
<td>6.0 ± 3.5</td>
</tr>
<tr>
<td><strong>Index of tissue edema</strong></td>
<td>0.13 ± 0.05</td>
<td>0.22 ± 0.05*</td>
<td>0.57 ± 0.06***</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td><strong>MPO (U/g)</strong></td>
<td>3.38 ± 0.51</td>
<td>11.67 ± 2.19***</td>
<td>4.33 ± 0.57***</td>
<td>2.33 ± 1.53*</td>
</tr>
<tr>
<td><strong>TNF-α (pg/mL)</strong></td>
<td>2.98 ± 0.25</td>
<td>15.82 ± 3.77***</td>
<td>10.41 ± 0.74**</td>
<td>8.15 ± 1.30*</td>
</tr>
<tr>
<td><strong>CAT (U/g)</strong></td>
<td>12.84 ± 0.87</td>
<td>7.74 ± 2.24**</td>
<td>9.07 ± 0.35*</td>
<td>11.68 ± 0.75</td>
</tr>
<tr>
<td><strong>SOD (U/g)</strong></td>
<td>0.31 ± 0.04</td>
<td>0.07 ± 0.02***</td>
<td>0.19 ± 0.05*</td>
<td>0.27 ± 0.03</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 in comparison with the normal group.
were not significantly different from those in the normal group (Table 4).

The much better therapeutic effect observed for QR microparticles might be attributed to the ability of chitosan microparticles to deliver sufficiently high QR concentrations to the inflamed colons. In contrast, the orally administered plain QR is absorbed from the upper gastro-intestinal tract leading to delayed onset of action and limited drug efficacy in colitis treatment.

4. Conclusion

The anti-inflammatory and antioxidant drug quercetin was loaded efficiently into chitosan-based colon-targeted delivery system to selectively target the inflamed colon. Quercetin loading capacity was fine-tuned and ranged from around 25–73%, indicating the ability of the system to encapsulate drugs of different therapeutic doses. The in vitro drug release profiles from the microparticles exhibited minimal drug loss in the gastric and intestinal simulated media and demonstrated rapid and selective drug release in the medium simulating pathological conditions in IBD colon. The therapeutic efficiency of the designed delivery system was examined using acetic acid-induced colitis rabbit model. The colon-targeted quercetin microparticles demonstrated higher therapeutic outcomes, as compared to the plain drug. These promising results confirm the utility of the designed colon-targeted system for IBD management.

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Author statement

Animal experiments were approved by the Institutional Animal Ethical Committee of the Faculty of Pharmacy, Assiut University and it adhered to the Guide for the Care and Use of Laboratory Animals, 8th Edition, National Academies Press, Washington, DC. The work described has been carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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