수의학 석사 학위 논문

다당류에 의해 나노캡슐화 된 퀘르세틴(Quercetin)을 통한 항암, 항염 그리고 항산화 활성의 개선 효과

Nano-encapsulated quercetin by polysaccharide enhances anticancer, anti-inflammation and anti-oxidant activities

2021년 8월

서울대학교 대학원

수의학과 수의생명과학 전공
(수의분자세포생물학)

문 현 진

Master's Thesis of Veterinary Medicine

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지도교수 백승준

이 논문을 수의학석사 학위논문으로 제출함

2021년 5월

서울대학교 대학원
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문 현 진

문현진의 수의학 석사 학위논문을 인준함

2021년 7월

위 원 장 이소영

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Abstract

Nano-encapsulated quercetin by polysaccharide enhances anticancer, anti-inflammation and anti-oxidant activities

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Dietary polyphenols including quercetin are secondary plant metabolites that have been reported to prevent many chronic diseases; however, the high hydrophobicity of quercetin results in low water solubility and bioavailability, which limits the therapeutic efficacy and impedes further applications in aqueous systems. Encapsulation may be a potential solution to the aforementioned problems. Some polysaccharides, including soluble soybean polysaccharides (SSPS) and chitosan, were widely used as polymeric backbones for the formation of nanoparticles. These nanoparticles are valuable drug delivery carriers. Thus, the objective of this study was to develop and subsequently evaluate the *in vitro* biological efficacy of nano-delivery systems of quercetin that utilize SSPS/chitosan carriers. I investigated the physicochemical property of encapsulated quercetin in SSPS with chitosan. Encapsulation of quercetin was confirmed using Dynamic light scattering (DLS), Zeta-potential (ζ), Fourier-transform infrared spectroscopy (FT-IR), Differential

scanning calorimetry (DSC), and Transmission electron microscopy (TEM). The anticancer, anti-inflammation and anti-oxidant activities of this nanoparticle was evaluated and confirmed based on cell culture system. The results showed that encapsulated quercetin exhibits better biological activity compared to free quercetin. Alongside the enhanced solubility of quercetin in an aqueous solution, my results illustrate the improved utility of this nanoparticle in the biomedical and food industry.

Keyword: Anticancer; Anti-inflammation; Anti-oxidant; Biopolymer nanoparticle; Chitosan; pH-driven method; Quercetin; Self-assembly; Soluble soybean polysaccharide

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Table of Contents

List of Figures

List of Tables

1. Introduction

Natural phytochemicals found in the diet have attracted increased attention due to their versatile biological activities. Quercetin is one of the most investigated phytochemicals and is abundant in numerous dietary plants such as celery, parsley, onions, and blueberry (Ulusoy and Sanlier 2020). Quercetin is believed to have a large number of health benefits, including anti-oxidant, anti-inflammatory, and anti-aging activities (Baksi et al. 2018; Li et al. 2016; Ramos et al. 2006; Zhao et al. 2017) (Fig. 1). More importantly, quercetin has shown promising prophylactic and therapeutic potential against cancers with its non-mutagenic property and relatively low toxicity. The potential chemotherapeutic effects of quercetin have been reported for various human cancer cells including colorectal cancer. These effects were achievable through inhibition of cell proliferation (Hashemzaei et al. 2017), promotion of cell cycle arrest (Yang et al. 2016), and induction of cell apoptosis (Zhang et al. 2015). Besides its anticancer activity, quercetin has been linked to the anti-obesity effect in humans and dogs (Kawasumi et al. 2018; Nabavi et al. 2015). Although the biological activities of quercetin are well understood, the high hydrophobicity of the quercetin significantly decreases its water solubility, thereby limiting its application in the food and biomedical industries. In addition, most phytochemicals exhibit rapid metabolism and elimination via glucuronidation as well as sulfation *in vivo*, which limits the bioavailability of quercetin and subsequently reduces its therapeutic efficacy (Wang et al. 2016). Therefore, improvement of solubility and bioavailability is of primary importance for the further development and application of quercetin (Luo, Wang, and Zhang 2020) (Fig. 2).

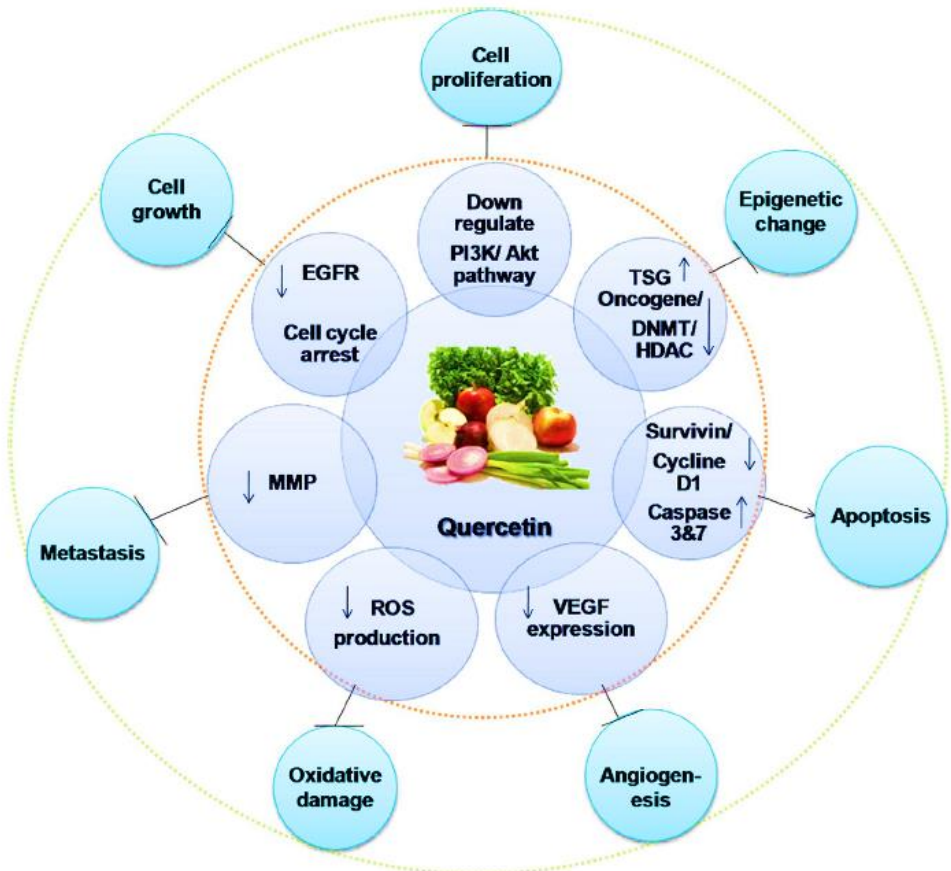

Figure 1. Anticancer activity of quercetin. (Baksi et al. 2018)

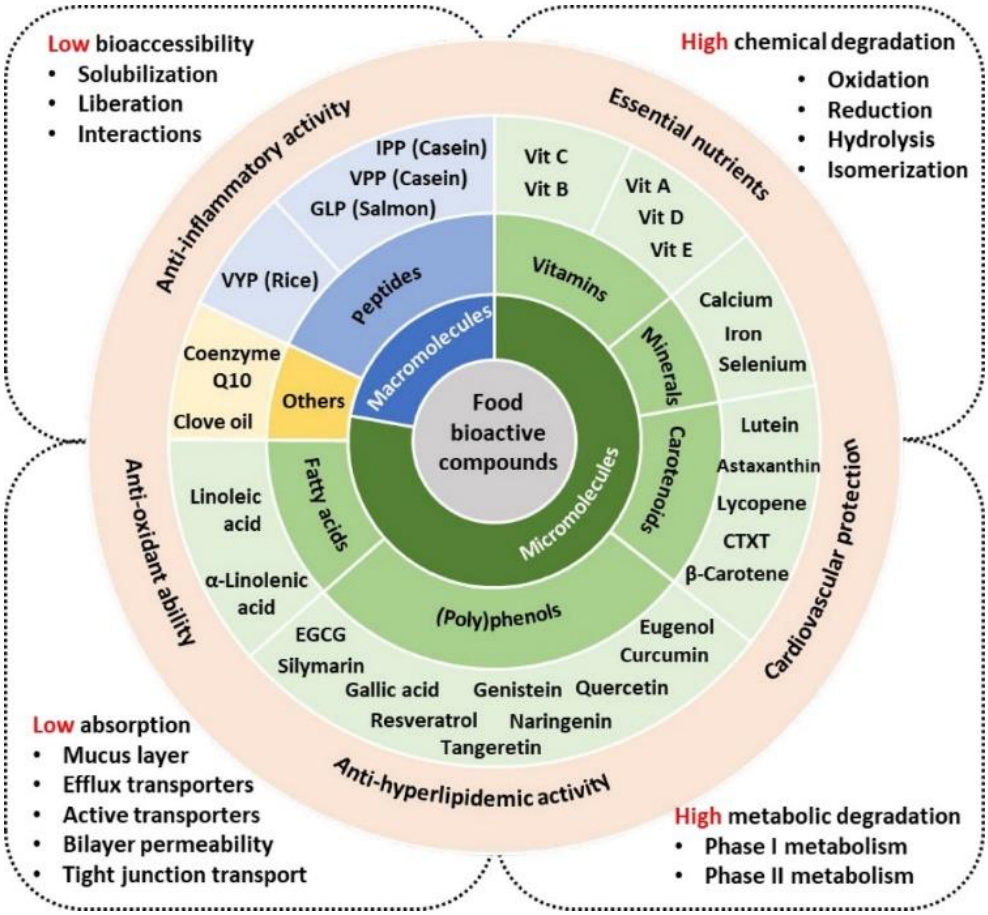

Figure 2. The need for biopolymer-nanoparticle to bioactive compounds. (Luo, Wang, and Zhang 2020)

Nanoparticles based on nanotechnology have emerged as efficient carriers for various biopharmaceutical agents including proteins and carbohydrates. In particular, polysaccharides are of great interest in the drug delivery field because of their various advantages, most notably: biocompatibility, biodegradability, low toxicity, and ease of modification (Barclay et al. 2019; Torres et al. 2019) (Fig. 3).

Natural polysaccharides are commonly obtained from several resources including animals, plants, and microbes (Jo and Lee 2020). Polysaccharides can be divided into two groups according to their charge (Li et al. 2018). Chitosan is a positively charged polysaccharide whereas, soluble soybean polysaccharide (SSPS) possess negative charge. Both are known to be safe, biocompatible, stable, hydrophilic, and biodegradable materials. Furthermore, chitosan and SSPS can be encapsulated with several hydrophobic compounds. SSPS is an anionic polysaccharide isolated from a soy protein byproduct. The detailed structure of SSPS has been previously determined using a stepwise enzymatic degradation (NAKAMURA et al. 2002). It was also reported that the major component of SSPS is the main backbone consisting of homogalacturonan and rhamnogalacturonan (Luo et al. 2019). SSPS has been used as a functional food ingredient in many different industrial applications and it has been suggested that SSPS could be used in flavor emulsions because of its high water solubility, low bulk viscosity, high-temperature stability, emulsifying properties, and ability to form strong interfacial films (Chivero et al. 2014). In addition, the negative charge of SSPS can promote the interaction with the positively charged chitosan. Chitosan is a biodegradable and bio-adhesive polysaccharide. It is a copolymer of glucosamine and N-acetyl-D-glucosamine linked by β (1,4) glycosidic bonds and it has been widely studied for several biological and biomedical applications, including those in the fields of nanotechnology (Divya and Jisha 2018).

The purpose of this study was to develop polysaccharide-based quercetin-loaded biopolymer nanoparticles that could have the potential to increase the bioavailability and therapeutic effects of quercetin. Both a quercetin-chitosan nanoparticle as well as a quercetin-SPSS nanoparticle complex was developed and observed for a variety of physicochemical characteristics. Of the following properties, we analyzed a pH-responsive profile, conducted a morphology analysis using transmission electron microscopy, and characterized the nanoparticle in terms

of size, zeta potential, and polydispersity index (PDI), DSC, and FT-IR.

My results indicate that quercetin with chitosan and SSPS provides better solubility and stability in cell-culture media/water, resulting in enhanced anticancer, anti-oxidant, and anti-inflammatory activities when compared to free quercetin.

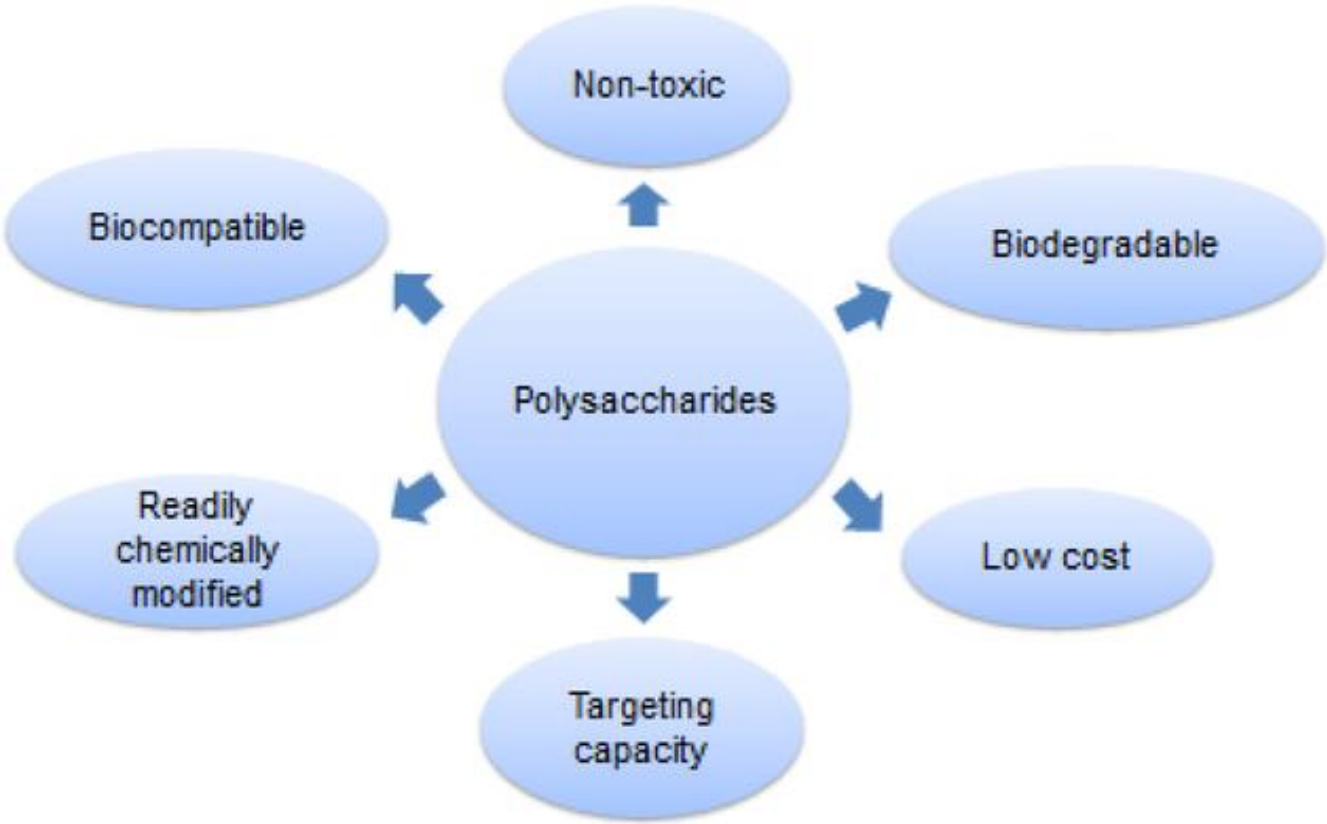

Figure 3. Key benefits of polysaccharides in drug delivery systems. (Barclay et al. 2019)

2. Materials and methods

2.1. Reagents

Soluble soybean polysaccharide (SSPS, SOYAFIBE S-CA700, Fuji Oil Co.Ltd, Osaka, Japan) was gifted by Namyung Co, Ltd (Seoul, Korea). Water-soluble chitosan (WSC) was obtained from Sokcho Biotech Co. (Sokcho-si, Gangwon-do, Republic of Korea). Quercetin (Q4951; purity ≥ 95%) and hydrogen chloride solution (HCL; 304174) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Biosesang (Gyeonggi-do, Korea) and sodium hydroxide (NaOH) was obtained from Duksan Pure Chemicals (Gyeonggi-do, Korea). Primary antibodies used in this study are as follows: iNOS (D6B6S) (13120S; Cell signaling; 1:1000), SP1 (PEP2) (sc-59; Santa Cruz Biotechnology; 1:500), p53 (DO-1) (sc-126; Santa Cruz Biotechnology; 1:500), GAPDH (0411) HRP (sc-47724; Santa Cruz Biotechnology; 1:1000), β-actin (C4) (sc-47778; Santa Cruz Biotechnology; 1:1000). The NAG-1/GDF15 antibody used was previously described (Baek et al. 2001).

2.2. Preparation of Quercetin nanoparticles (QN)

Quercetin nanoparticles were prepared using the pH-driven encapsulation procedure with some minor modifications (Pan et al. 2018). Briefly, SSPS and chitosan were dissolved in deionized water overnight at 4 °C. The 2% (w/w) SSPS solution was adjusted to pH 12.0 with 4.0 M NaOH solution and quercetin was added at the indicated concentrations of 0.25, 0.5, 1, and 2 mg/ml. SSPS solution without quercetin was used as a negative control. After stirring for 30 min at room temperature, the mixture was adjusted to pH 7.0 using 1.0 M HCl, and then it was stirred for an additional 5 min. Next, 0.05% (w/w) chitosan solution was added to the mixture so that it constituted 15% of the total mixture volume and the new mixture was stirred for an additional 1 h. The final mixture was centrifuged at 2920 g for 10 min at RT to remove precipitants with unloaded quercetin. The supernatant was freeze-dried (FDS8508, IIshin Co., Seoul, Korea) at -80 °C and packed into plastic tubes tightly for future experiments.

2.3. Encapsulation efficiency (EE) and loading capacity (LC)

To measure the quercetin concentration in QN, a certain amount of quercetin in the precipitate was dissolved in methanol (106009; Merck KGaA, Darmstadt, Germany) and measured a concentration of quercetin using a UV-VIS spectrophotometer (Multiskan Sky Microplate Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). The amount of quercetin in the precipitate was based on the absorbance of the solution at 415 nm. The concentration was calculated by referring to a standard curve established from standard quercetin solutions in methanol. The EE and LC are calculated by the following equation below.

$$\text{EE (\%)} = \frac{\textit{The total amount of Quercetin (mg)} - \textit{amount of Quercetin in precipitate (mg)}}{\textit{Total amount of Quercetin (mg)}} \times 100$$

$$\text{LC (mg/g)} = \frac{\textit{The total amount of Quercetin (mg)} - \textit{amount of Quercetin in precipitate (mg)}}{\textit{Total amount of SSPS (g)}}$$

2.4. Determination of particle size and Zeta-potential (ζ)

Particle size and zeta-potential were measured by Zetasizer Nano-ZS90 (Malvern Instruments Ltd, Malvern, UK). The sample was diluted 20 times using deionized water. Each sample was analyzed in triplicate to determine the average particle size and zeta-potential.

2.5. Transmission electron microscopy (TEM)

The morphology of free SSPS (before pH cycle) and quercetin nanoparticles was measured by TEM. The sample was diluted 10 times with deionized water and subsequently dropped on glow-discharged carbon film on a 400-mesh copper grid stained with uranyless EM stain solution (Electron Microscopy Sciences; EMS, Hatfield, PA, USA). Samples were captured with LIBRA 120 operating at 120 kV (Carl Zeiss, Germany).

2.6. Differential scanning calorimetry (DSC)

Thermal properties were measured using a differential scanning calorimeter (DSC 4000, Perkin Elmar, Inc., Waltham, MA, USA). In brief, freeze-dried samples were used for DSC analysis. All samples (SSPS 15.3 mg, free quercetin 10.2 mg, chitosan 15.5 mg, SSPS after pH cycle 4.6 mg, SSPS after pH cycle + chitosan 7.8 mg, quercetin nanoparticle without chitosan 4.4 mg, and quercetin nanoparticle with chitosan 5.2 mg) were placed in an aluminum pan and hermetically sealed. The samples were heated from 30 °C to 350 °C at a rate of 10 °C/min after holding for 1 min at 30 °C. Onset temperature was determined from the glass transition by heat flow. Peak temperature was determined from the maximum heat flow and the enthalpy change was calculated from the peak of the curve. An empty aluminum pan was used as a reference.

2.7. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed by Nicolet 6700 (Thermo Fisher Scientific, USA). The samples were measured using a wavenumber of 4000-600 cm^{-1} with a resolution of 8 cm^{-1} and the recorded result was the aggregate of 32 scans.

2.8. In vitro anti-oxidant activity assay

Samples were dissolved in distilled water and used for the following experiments. The quercetin concentrations in free quercetin (FQ) and quercetin nanoparticle (QN) used in this experiment were 125, 62.5, 31.25, 15.625, and 7.8125 μg/ml. The concentrations of SSPS with chitosan were 2, 1, 0.5, 0.25, and 0.125% (w/w). DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate, #D9132; Sigma-Aldrich, USA) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, #10102946001; Sigma-Aldrich, USA) were used for the radical scavenging assay as previously described (Lertpatipanpong et al. 2020). The absorbance was measured using the Multiskan™ FC Microplate photometer (Thermo Fisher Scientific, USA) at 492 nm and 740 nm for DPPH and ABTS, respectively. L-ascorbic acid (Vitamin C) (#A0537, TCI, Tokyo, Japan) was used as a standard in both assays. To determine anti-oxidant capacity of the samples, the percentage of radical scavenging effect was

considered using the following equation:

$$\text{ABTS (or DPPH) scavenging activity (\%)} = \left[\frac{Abs\ of\ control - (Abs\ of\ Sample - Abs\ of\ blank)}{Abs\ of\ Control}\right] X\ 100$$

The anti-oxidant activities were expressed as vitamin C equivalent anti-oxidant capacity (VCEAC) in mg/g dried weight of the sample. The IC_{50} value indicates half of the concentration of the sample that can scavenge 50% of the free radicals of DPPH.

2.9. Cell culture

Human colon cancer HCT-116 and human osteosarcoma U2OS cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, USA). Mouse macrophage Raw264.7 and human fibroblast CCD-986sk cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. HCT-116, U2OS, and Raw264.7 cells were purchased from the American Type Culture Collection (ATCC), whereas CCD-986sk cells were purchased from the Korean Cell Line Bank (Seoul, Korea). All cells were incubated at 37 °C under 5% CO_2.

2.10. Dual-Luciferase assay

Dual-Luciferase assay was performed using the Dual-Luciferase Reporter Assay kit (Promega, WI, USA) according to the manufacturer's protocol. Briefly, HCT-116 cells were seeded into a 24-well plate and incubated for 24 h. The vectors including pARE-luc (Yoo et al. 2020) were co-transfected with pRL-null using Polyjet™ in vitro DNA transfection reagent (SignaGen, Frederick, MD, USA). After transfection, SSPS-chitosan, FQ, and QN dissolved in media were treated for 24 h. The final concentration of quercetin in FQ and QN was 50 μg/ml. The relative luciferase activity was measured using the Synergy™ HTX multi-mode microplate reader (Biotek Instruments, Winooski, VT, USA).

2.11. Cell proliferation assay

Free quercetin dissolved in DMSO or media, QN and SSPS-chitosan dissolved in media were prepared for cell treatment. HCT-116, U2OS, CCD-986sk cells were cultured on a 96-well plate and treated with the indicated compound in complete medium for 72 h. Cell proliferation assays were then performed using CellTiter 96® AQ_{ueous} One Solution (Promega, USA) according to the manufacturer's instructions. After 72 h incubation, 20 μL of One Solution reagent was added to each well and cells were incubated for an additional 1 h at 37 °C. Cell viability was estimated by measuring the absorbance at 490 nm with the Multiskan FC spectrophotometer (Thermo Fisher Scientific, USA).

2.12. Apoptosis assay

HCT-116 cells were seeded on a 6-well plate and treated with FQ, SSPS-chitosan, or QN dissolved in serum-free media. No-treatment was used as control. After treatment for 24 h, cells were stained with FITC/Annexin V Apoptosis Kit with PI (#640914; BioLegend, San Diego, CA, USA) according to the manufacturer's protocol and they were analyzed using the SH 800 Cell sorter (Sony Biotechnology Inc., San Jose, CA, USA). The raw data were analyzed with the FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.13. Western blot analysis

After incubation with the compounds, Cells were harvested using RIPA buffer (GenDEPOT, Katy, TX, USA) containing proteinase and phosphatase inhibitors. Protein concentration was measured using a BCA assay kit (Thermo Fisher Scientific, USA). The same amounts of protein (30 μg) were separated by Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes (GVS Filter Technology, Sanford, ME, USA). The membrane was blocked in Tris-buffered saline with 0.05% Tween 20 (TBS-T) containing 5% (w/v) skim milk for 1 h at RT and then incubated with specific primary antibodies diluted in TBS-T buffer containing 5% skim milk at 4 °C, overnight. The membrane was washed thrice with TBS-T buffer for 10 min. Next, they were

incubated with the appropriate secondary antibodies (1:5000) diluted in TBS-T buffer containing 5% skim milk for 2 h at RT and then they were washed again. The enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, USA) was used alongside the Alliance Q9 Advanced system (UVTEC CAMBRIDGE, Cambridge, England, UK) to visualize protein expression.

2.14. Statistical analysis

All experiments were performed on at least three independent experiments. Statistical analysis was performed using Microsoft Office Excel and GraphPad Prism 8. Values are expressed as mean ± SD or SEM and data was analyzed using a student's t-test. P-value (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) was considered to be statistically significant.

3. Results

3.1. Preparation of Quercetin Nanoparticles

Phytochemicals including quercetin contain multiple hydroxyl groups and exhibit pH-dependent solubility. They are usually insoluble in water at a neutral pH but soluble at an alkaline pH when their hydroxyl groups are deprotonated. In the previous report, they developed self-assembled curcumin-SSPS using a pH-driven method (Pan et al. 2014) and damnacanthal using the deoxycholic and poly(ethylene glycol) methyl ether grafted chitosan (DCA-CS-mPEG) (Sukamporn et al. 2017).

In this study, I applied both SSPS and chitosan to encapsulate quercetin and subsequently characterized these nanoparticles based on their physicochemical and biological activities. At pH 7.0, SSPS is dispersed in water as single molecules. Upon increasing pH to 12.0, quercetin is dissolved as clear solution with hydrophobic interactions occurring between the quercetin and SSPS. When the pH is decreased to 7.0 at initiation, the quercetin is re-protonated and becomes insoluble, in the meantime, it also aggregates with SSPS to form biopolymer nanoparticles. By addition of a small amount of chitosan, which has an opposite charge to SSPS, leads to improvement of the encapsulation stability of nanoparticles probably due to the electric charge interaction between the chitosan and SSPS (Fig. 4).

When quercetin was encapsulated using only SSPS, the interaction between quercetin and SSPS appeared to be weak, and quercetin was gradually released from the SSPS and then precipitated after day 1. Nevertheless, after chitosan was added, the encapsulation stability was prolonged and quercetin was well complexed with SSPS-chitosan for as long as 20 days (Fig. 5).

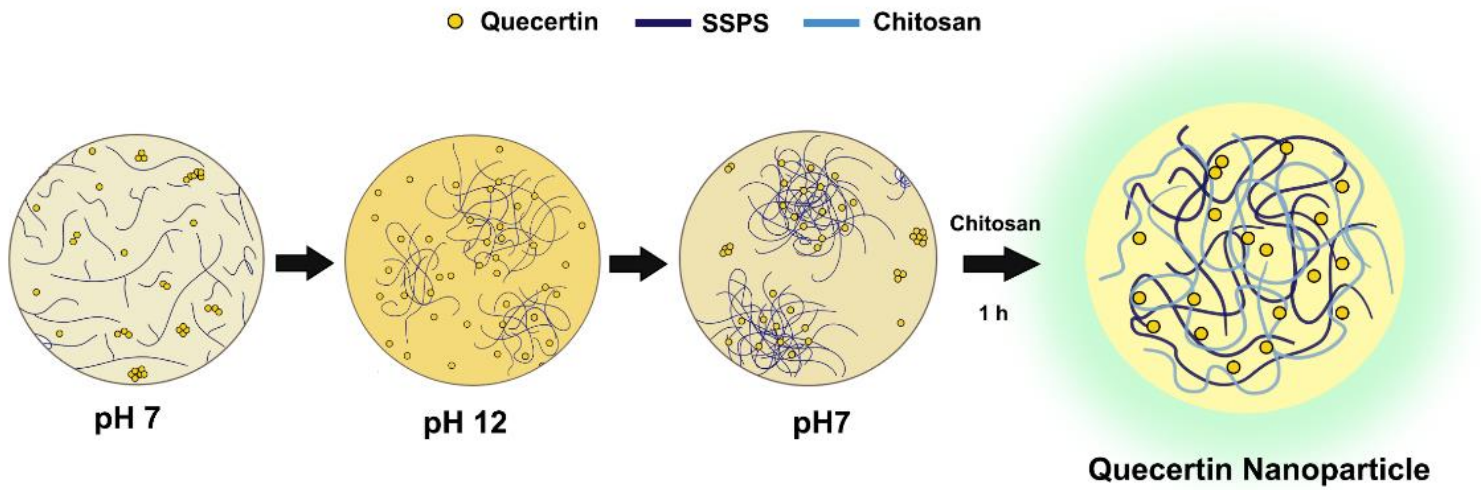

Figure 4. Schematic diagram of quercetin encapsulated in soybean polysaccharide (SSPS) with chitosan by the pH-driven method.

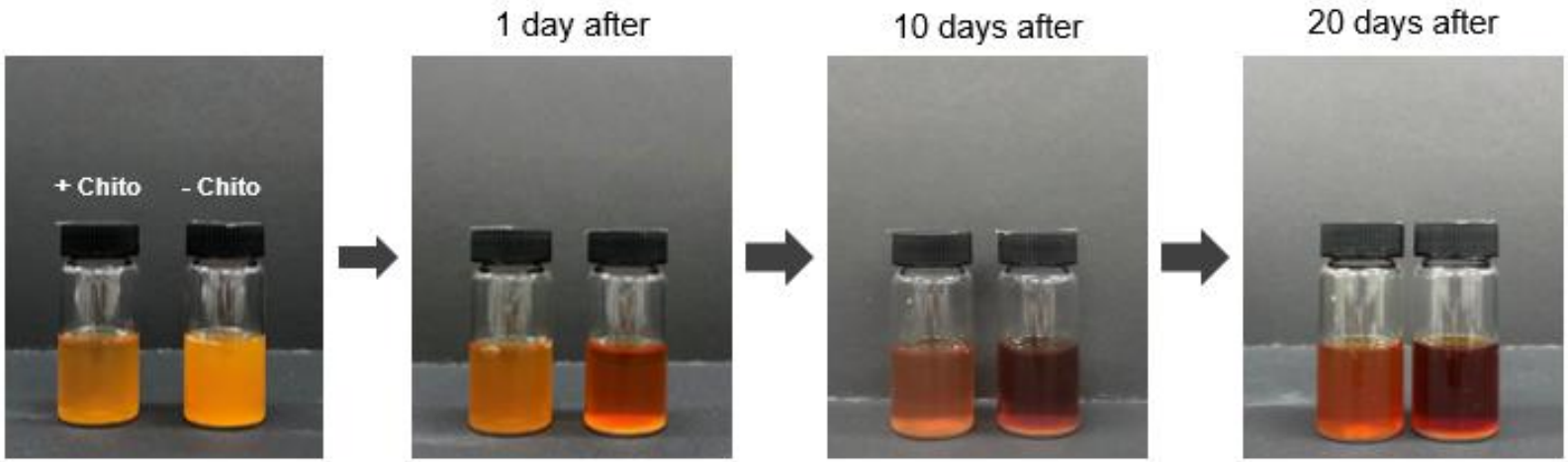

Figure 5. Comparison between quercetin encapsulated in SSPS with chitosan and without chitosan for 20 days.

3.2. Characterization of Quercetin Nanoparticles

Following centrifugation of the quercetin nanoparticle with SSPS and chitosan, a transparent yellow-brown colored solution was obtained. Free quercetin in water was translucent when initially mixed, but over time it precipitated (Fig. 6).

3.2.1. Particle size, Zeta-potential and PDI index

To identify nano-size particles, TEM analysis was conducted and the image confirmed the physical structure changes during encapsulation of quercetin (Fig. 7). When SSPS was initially dispersed in water, the SSPS appeared to be as mixture which composed of large and small single substances. However, following quercetin encapsulation and combination with chitosan, the structure assumed a sphere shape due to self-aggregation.

The data from dynamic light scattering (DLS), the most often used characterization method for nanoparticles, indicated the average size of quercetin nanoparticles is 24.438 ± 4.33 nm. This result was concordance with that obtained by the TEM image (Fig. 8).

In addition, a comparison of nanoparticle properties with and without chitosan indicated that adding chitosan provided better complexity in the SSPS-quercetin mixture. The mean size of the particle slightly increased when chitosan was added; however, it was still in the nanoparticle size range. Upon the addition of chitosan, the polydispersity index (PDI) value was decreased from 0.8 to 0.59, thereby confirming increased stability in the monodisperse particle form (Table 1).

The zeta potential data showed the surface charge of nanoparticles. SSPS-quercetin nanoparticles have a negative charge following a pH cycle. We confirmed that the absolute value was improved when chitosan was added, which indicates chitosan may increase the stability of the complex (Table 1).

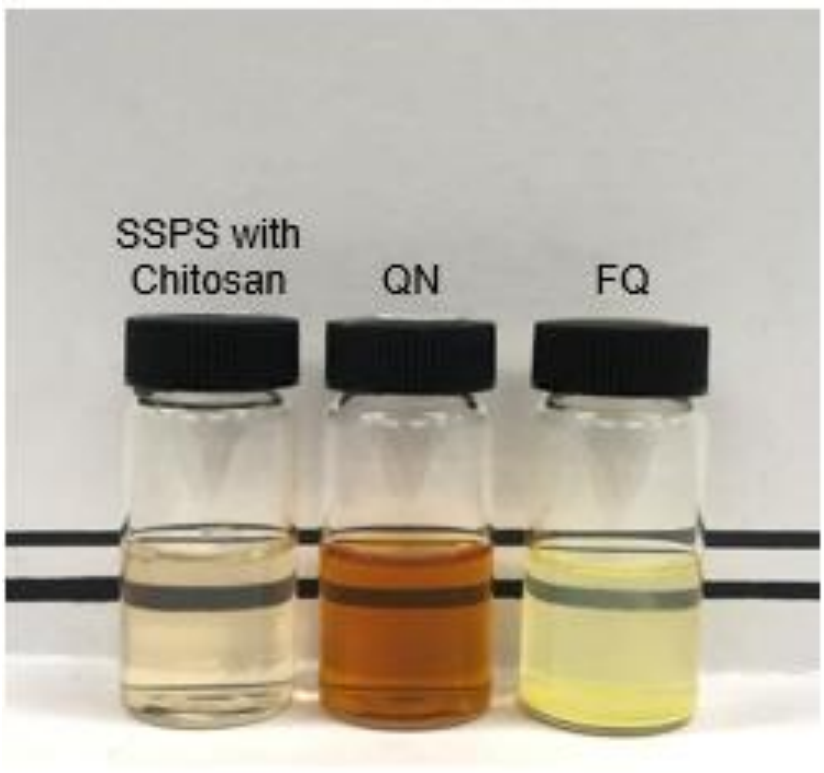

Figure 6. Appearance of SSPS with chitosan, quercetin nanoparticles (QN) and free quercetin (FQ) in deionized water.

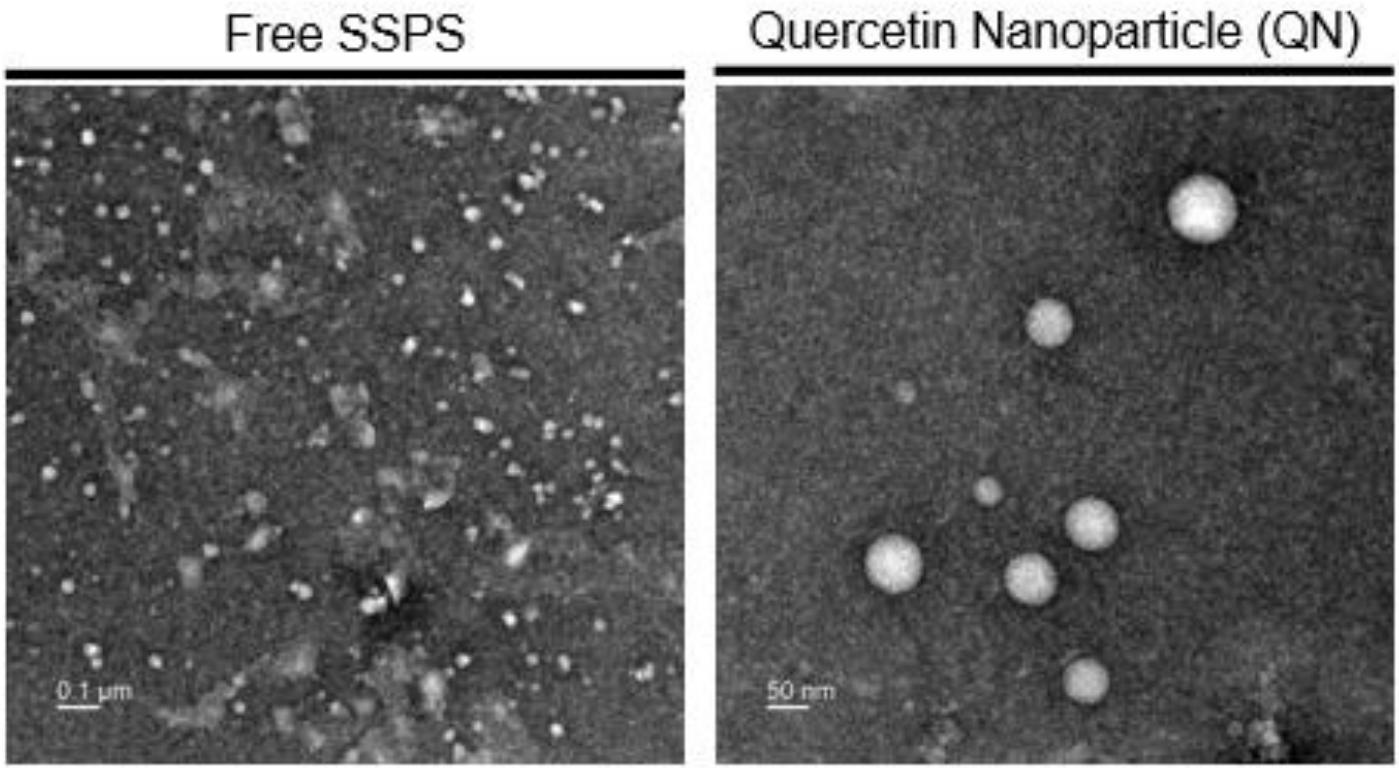

Figure 7. TEM images of Free SSPS (before pH cycle) and quercetin nanoparticles (QN). Free SSPS (before pH cycle) and quercetin nanoparticle solution was prepared as described in the Methods section.

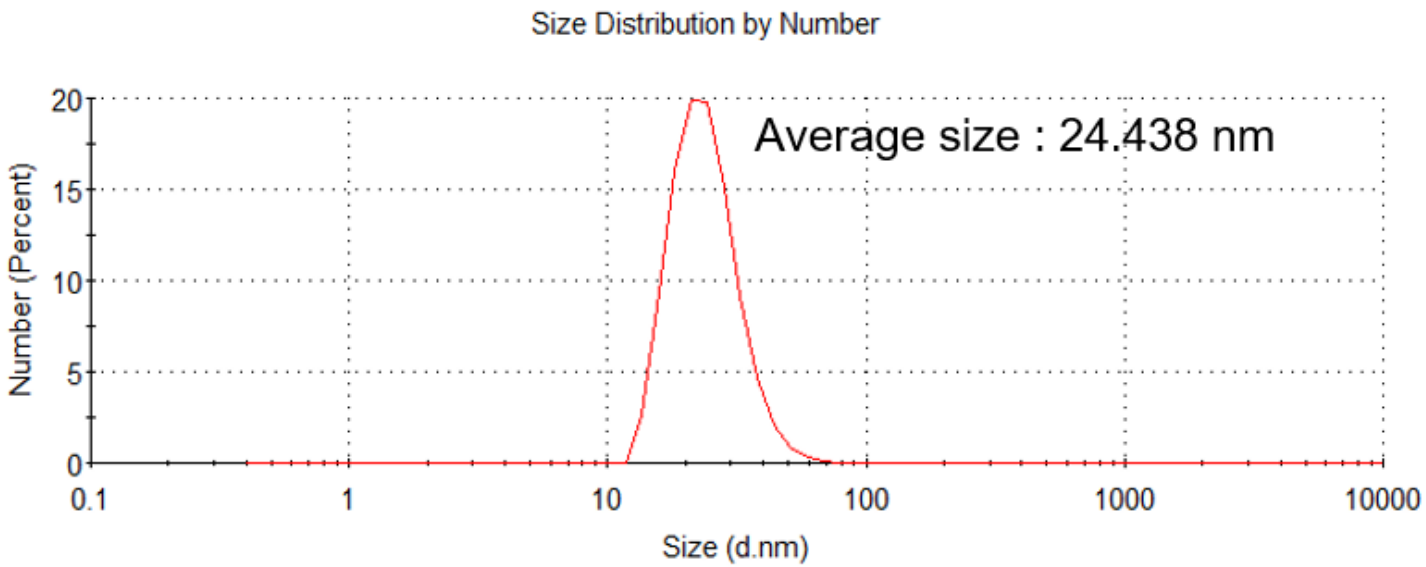

Figure 8. Particle size distribution of quercetin nanoparticles as measured by dynamic light scattering.

Table 1. Comparison of Quercetin nanoparticles with and without chitosan

	Mean size (nm)	PDI	ζ-potential
QN without Chitosan	20.24 ± 5.71	0.80 ± 0.01	-17.5 ± 0.85
QN with Chitosan	24.44 ± 4.33	0.59 ± 0.02	-24.5 ± 1.75

Numbers are mean ± standard deviation (n=3).

3.2.2. Encapsulation efficiency and loading capacity

I also measured the encapsulation efficiency (EE) and loading capacity (LC) of quercetin nanoparticles. Various concentrations (0.25, 0.5, 1.0, and 2.0 mg/ml) of quercetin were added in SSPS solution and the EE and LC of each concentration were measured (Fig. 9). The EE was over 98% in the 0.25, 0.5, and 1.0 mg/ml concentrations; however, the EE decreased (76.9 ± 16.46) at the highest dose level (2.0 mg/ml). This may occur because the amount of quercetin exceeded supersaturation levels and the encapsulation state was unstable when quercetin was added to the SSPS dispersion at a concentration of 2.0 mg/ml or more. The LC was from 12.32 to 76.79 mg (quercetin)/g (SSPS). For the subsequent experiments, we used the 0.5 mg/ml concentration since it has the highest EE with the highest zeta potential (data not shown), compared to 0.25, 1.0, and 2.0 mg/ml of quercetin.

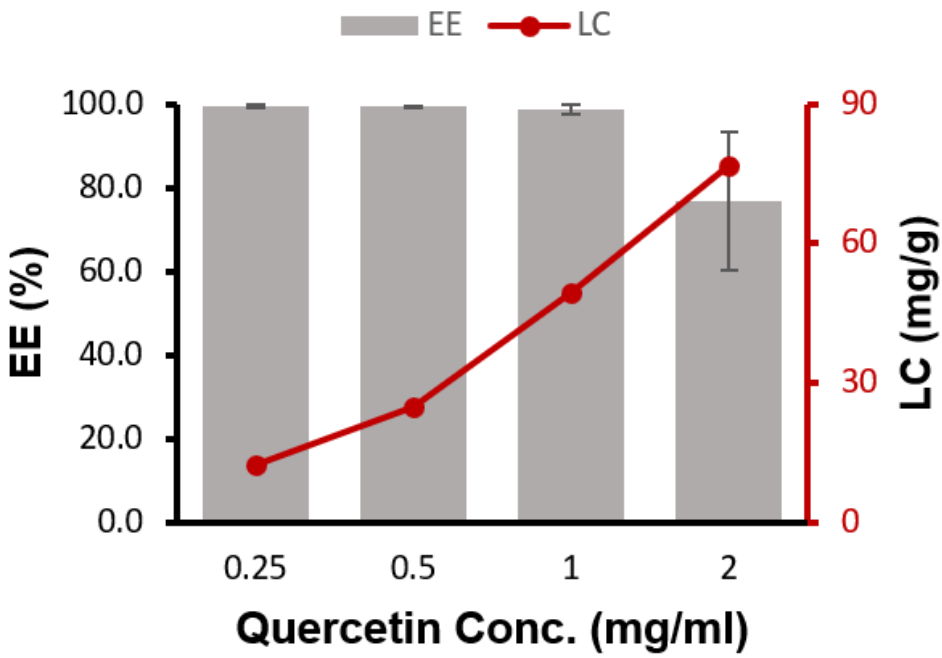

Figure 9. Encapsulation efficiency (EE) and loading capacity (LC) of quercetin nanoparticles with different concentrations (0.25, 0.5, 1 and 2 mg/ml). Data are shown as mean ± STD (n=3).

3.2.3. DSC analysis of quercetin nanoparticle

To measure the compatibility of quercetin with SSPS and chitosan, a DSC measurement was performed (Fig. 10). The free SSPS, free quercetin, and chitosan showed a characteristic sharp endothermic peak. SSPS (red line) has an endothermic peak at 198 °C and water-soluble chitosan (mustard line) has three endothermic peaks at 193 °C, 213 °C and 313 °C. Free quercetin (orange line) has two endothermic peaks at 127 °C and 324.7 °C, which corresponds to dehydration and melting, respectively (Pool et al. 2012). Conversely, their characteristic peak was disappeared in QN and its control, thereby confirming the loss of the crystalline structure and encapsulation of quercetin in a polysaccharide matrix.

The sample without chitosan (SSPS after pH cycle, QN without chitosan) and with chitosan (SSPS after pH cycle chitosan, QN with chitosan) showed two endothermic peaks. The first endotherm is observed with a broad endothermic peak in all four samples (green, blue, brown, and violet lines), which might be the phase change of glass transition. However, the first endotherm in the QN with chitosan sample (violet line) is obtained at onset temperature (at 133.9 °C) and exhibited a broader peak endotherm at 179.7-224.4 °C, when compared to the mixture of SSPS after pH cycle with chitosan (blue line). A second endotherm is obtained in the temperature between 252 °C and 270 °C. Simultaneously, the second endotherm enthalpy of SSPS/chitosan (blue line) greatly increases than QN with chitosan (violet line) from 9.4 J/g to 73.6 J/g. These results suggest that higher temperature endotherm is associated with melting of SSPS, quercetin, and chitosan. Moreover, the enthalpy of the second endotherm of QN with chitosan (violet line) exhibited a higher value (73.6 J/g) than QN without chitosan (42.2 J/g) (brown line). This may indicate that the addition of chitosan makes QN more stable, barring any thermal transition due to the higher degree of crystallinity in the QN.

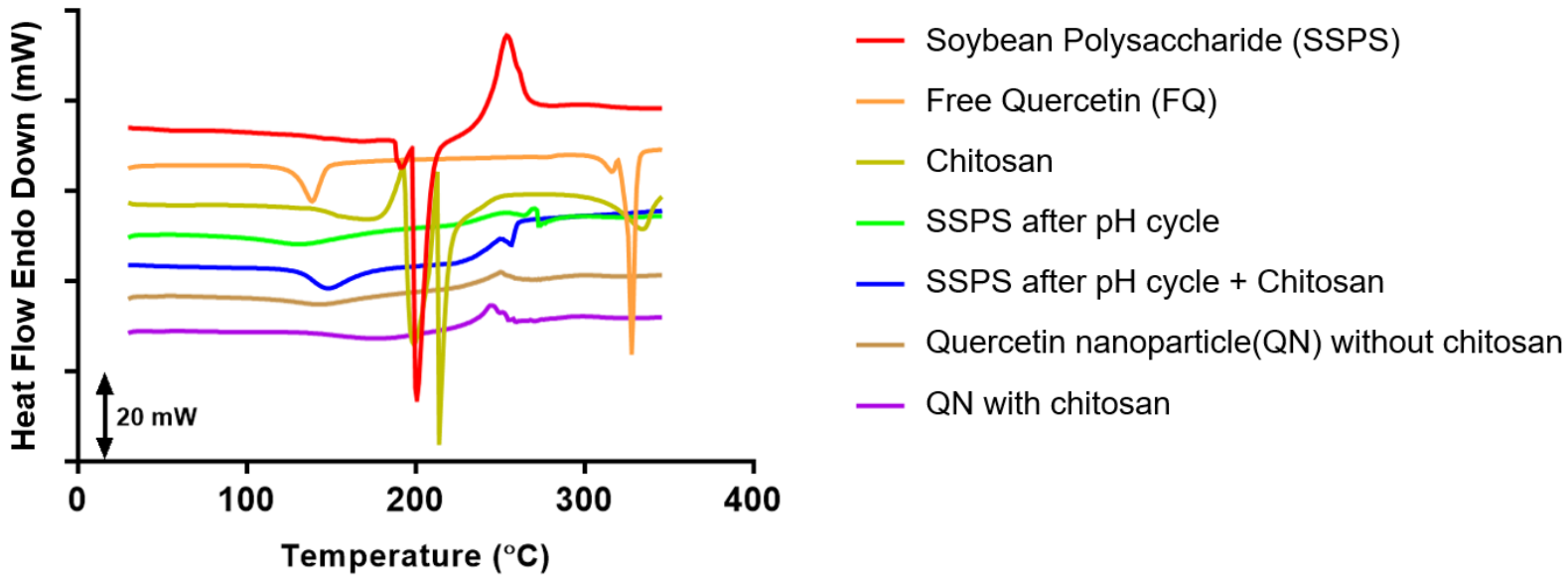

Figure 10. Differential scanning calorimetry (DSC) thermogram of Soybean soluble Polysaccharide (SSPS), Free Quercetin (FQ), Chitosan, SSPS after pH cycle, SSPS after pH cycle + chitosan, Quercetin nanoparticle (QN) without chitosan and QN with chitosan.

3.2.4. FTIR analysis of quercetin nanoparticle

An FTIR measurement was performed to examine the potential molecular interactions between quercetin and polymer (Fig. 11).

The FTIR spectrum of SSPS showed an absorption band at 3316.45 cm^{-1} related to hydroxyl stretching vibration and a weak absorption band at approximately 2931.16 cm^{-1} related to the C-H stretching vibration of CH_2 groups. The absorption at 1612.69 cm^{-1} corresponded to the stretching vibration of the CHO and C=O bonds. Finally, the absorption bands at 1412.21 cm^{-1} could be the deforming vibrations of C-H bonds (Mao et al. 2013). The FTIR absorption peak for SSPS after the pH cycle changed from 2931.16 to 2917.24 and 2849.53 cm^{-1}, and from 1612.69 to 1570.20 cm^{-1} when compared with free SSPS. This shift indicates that SSPS self-aggregates as it goes through the pH cycle and a structural change occurs due to glycosidic interactions between them.

The chitosan spectrum presents characteristic peaks at 3267.83 cm^{-1} related to the stretching vibration of $-NH_2$ and -OH groups as well as a peak at 2918.10 cm^{-1} related to the stretching vibration of the C-H bond. Other peaks appeared at 1613.03 and 1509.75 cm^{-1} corresponding to the amide group (Huang et al. 2012). In the FTIR peak of SSPS after pH cycle + chitosan sample, the peaks representing the amide group of chitosan showed a new peak from 1613.03 and 1509.75 cm^{-1} to 1560.21 cm^{-1}. In the SSPS after pH cycle, the addition of chitosan weakened the peak in the 2900 range. This result suggests there might be electrostatic interactions between the two polysaccharides.

In pure quercetin crystal, many specific peaks could be detected that could confirm the quercetin chemical structure (Porto et al. 2018). The broadband at 3302.82 cm^{-1} refers to O-H stretching of the phenolic hydroxyl bonds. The intensity at 1667.82 and 1610.67 cm^{-1} are related to a C=O carbonyl group. The characteristic band of C=C aromatic bonds stretching at 1559.02, 1512.31 cm^{-1} was also observed. The presence of the band at 1313.47 to 1162.83 cm^{-1} confirms C-O stretching. Finally, the peak at 932.99 cm^{-1} represents the C-H bending vibration of aromatic groups. However, the transmission peaks at these specific peaks could not be observed in QN, which indicates that quercetin was well encapsulated from SSPS following the pH cycle. Moreover, weak peaks appeared in the 2900 cm^{-1} and 1520 cm^{-1} range for

QN samples with and without chitosan, proving that the quercetin has hydrophobic interaction with SSPS.

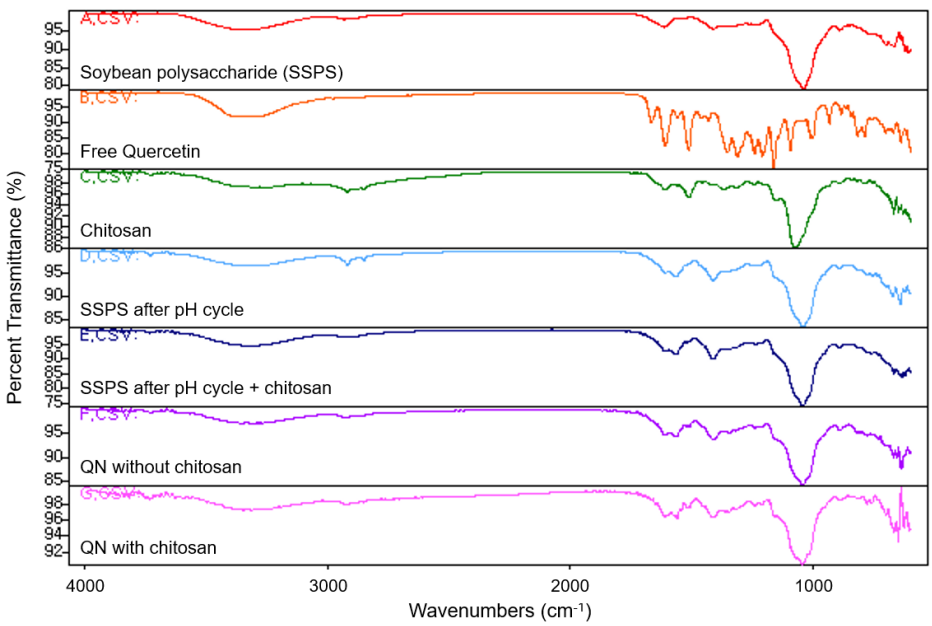

Figure 11. FTIR spectrum of SSPS, FQ, Chitosan, SSPS after pH cycle, SSPS after pH cycle + chitosan, QN without chitosan, QN with chitosan.

3.3. The anti-oxidant and anti-inflammation activity of quercetin nanoparticles

It is known that quercetin affects anti-oxidant and anti-inflammatory activities (Li et al. 2016; Zhang et al. 2011). Thus, the anti-oxidant and anti-inflammatory activity of QN was investigated.

DPPH and ABTS radical scavenging activities were measured to determine the anti-oxidant effect of FQ, SSPS with chitosan, QN, and ascorbic acid (VitC) dissolved in distilled water at different concentrations (Fig. 12). The VCEAC, the vitamin C equivalent anti-oxidant capacity of FQ and QN were calculated as 707.21 ± 33.33 and 743.58 ± 1.50 mg/g dried weight, respectively. Whereas, as a negative control, the SSPS with chitosan at the indicated concentration was found to be insufficient to vanish the ABTS oxidant radicals, demonstrating the low capacity to scavenging the oxidant compound (VCEAC of SSPS with chitosan is 71.68 ± 13.37 mg/g dried weight) (Fig. 12A). Furthermore, I also confirmed that DPPH scavenging activity of FQ and QN are increased with concentration, similar to ABTS scavenging activity results. However, in the case of QN, it required a higher concentration to reduce the oxidant radicals than FQ because its scavenging effect is less than FQ (Fig. 12B). For the IC_{50} value of VitC, FQ, and QN were 77.30 ± 0.81, 93.84 ± 3.39, and 174.96 ± 2.86 μg/ml, respectively.

To further confirm the anti-oxidant activity, I transfected a luciferase construct containing anti-oxidant response element (ARE), an NRF2 responsive reporter, into HCT-116 cells (Fig. 13). The luciferase activity was increased after treatment of FQ, SSPS with chitosan, QN compared to the negative control (untreated cells; media). Although quercetin is known to be insoluble in water, it has been reported that a very small amount of quercetin can be dissolved in water (Srinivas et al. 2010). Moreover, because of their lipophilic property, flavonoids tend to accumulate in biological membranes and influence their functioning (Tarahovsky et al. 2014). It is likely that a small amount of the quercetin crystal might be dissolved in media, and penetrate the cell membrane, thereby resulting in increased luciferase activity. SSPS and chitosan are also known to have an anti-oxidant effect (Ngo and Kim 2014), this confirm by the luciferase activity was slightly increased compared to the negative control. In the case of QN, the luciferase activity increased as twice of the negative

control. This result indicates QN has anti-oxidant effect through the activation of the NRF2 protein followed by the activation of the ARE promoter.

Anti-oxidant activity is linked to anti-inflammation activity (Arulselvan et al. 2016). To confirm the anti-inflammation activity of QN, I treated FQ, SSPS with chitosan, QN dissolved in media into Raw264.7 macrophage cells, followed by the addition of 1 μg/ml of LPS (Fig. 14). Western blot data showed the increasing of the iNOS protein expression levels after treatment of LPS; however, FQ and QN treatment were shown to decreasing the iNOS protein expression levels, which indicating the quercetin nanoparticles were effective nanoparticles since they demonstrated the resemble similar anti-oxidant and anti-inflammation activity as free quercetin crystals.

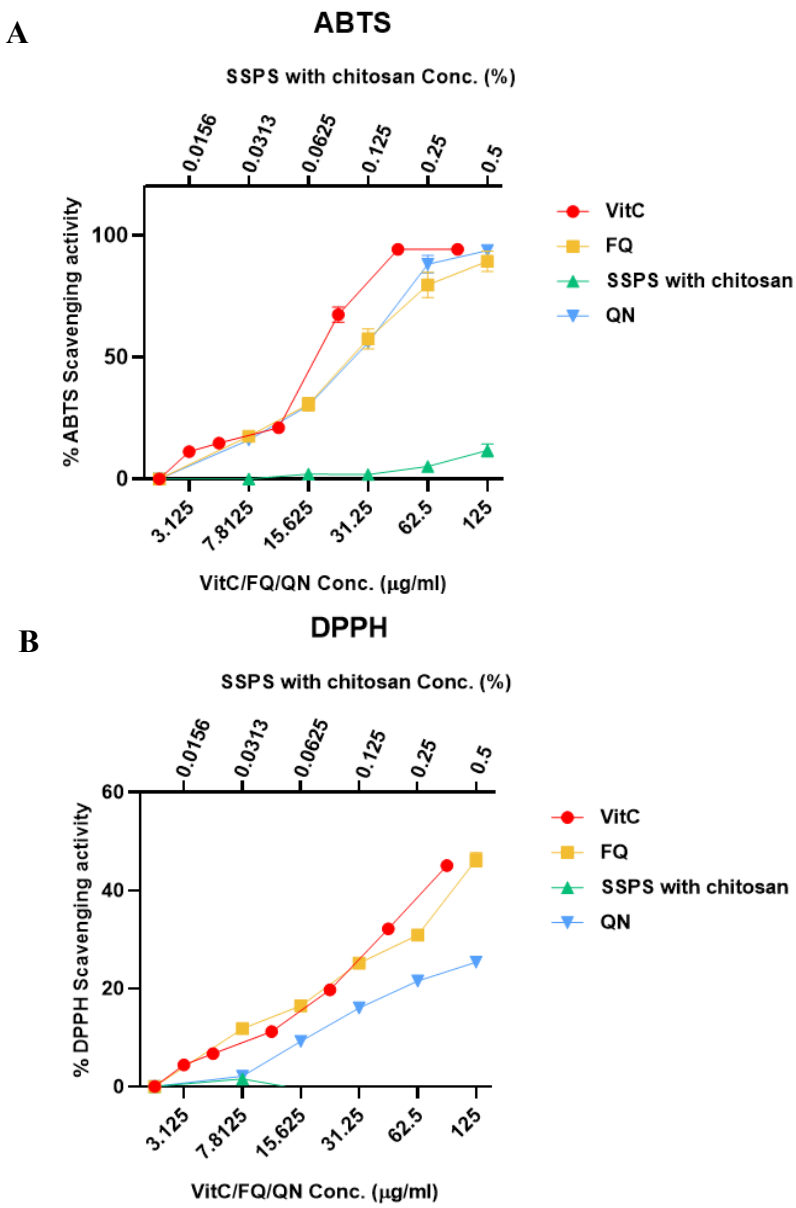

Figure 12. Anti-oxidant activity of FQ, SSPS with chitosan and QN. (A) ABTS radical scavenging ability of the FQ, QN, SSPS with chitosan (for control). SSPS with chitosan concentration was used as 0.0156/0.0313/0.0625/0.125/0.25/0.5% (w/w), the same amount as dose concentration used in QN. Quantification of the result from three independent experiments (n=3) is shown as mean ± SEM. Ascorbic acid (VitC) was used as a positive control. (B) DPPH radical scavenging ability of the FQ, QN, SSPS with chitosan (for control). SSPS with chitosan concentration was used the same as (A). Quantification of the result from three independent experiments (n=3) is shown as mean ± SEM. Ascorbic acid (VitC) was used as a positive control.

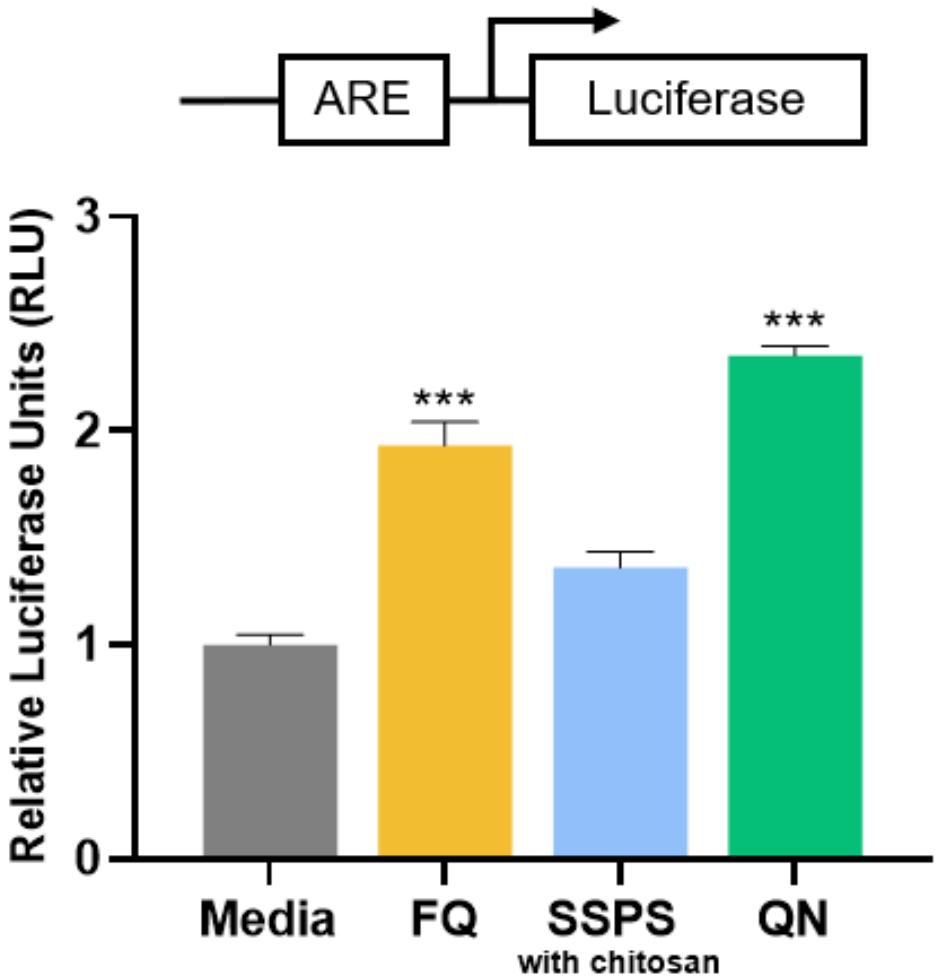

Figure 13. The ARE-luciferase activity of FQ, SSPS with chitosan and QN. HCT-116 cells were co-transfected with pARE (Anti-oxidant response element)-luciferase vector and pRL-null, followed by the treatment of FQ, SSPS with chitosan (for QN control), and QN. The media treatment was used as the control for FQ, whereas SSPS with chitosan was used as the control for QN. After 24 h treatment of indicated compounds, luciferase activity was measured. The quercetin concentration in both FQ and QN was 50 μg/ml. Results are expressed as mean ± SD (n=3). ***$P < 0.001$ compared with each control.

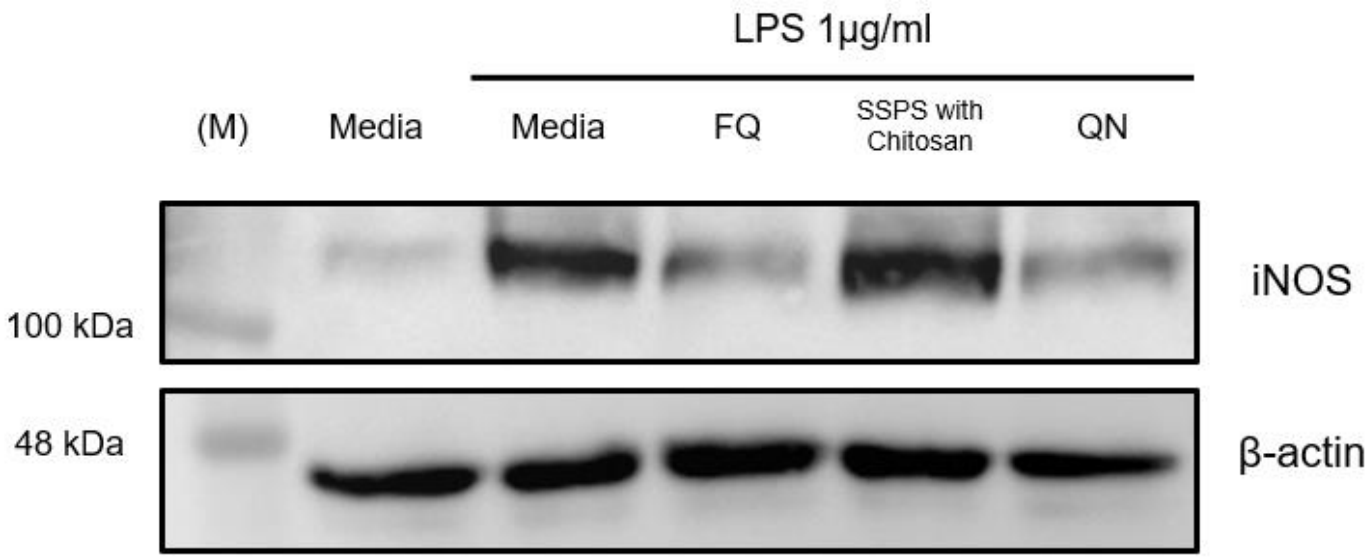

Figure 14. Western blot analysis of RAW264.7 macrophage cells treated with FQ, SSPS with chitosan (control for QN), and QN, followed by the treatment of 1 μg/ml LPS. The media treatment was used as control. The quercetin concentration in both FQ and QN was 50 μg/ml. M represents protein size marker.

3.4. Anticancer activity of quercetin nanoparticles

To confirm the solubility of free quercetin and quercetin nanoparticles in media, I dissolved FQ crystal and QN in cell culture media, treated them into HCT-116 and U2OS cells, and observed the cells under a microscope following a 24 h incubation period (Fig. 15). In the case of free quercetin, the result confirmed that most of quercetin cannot dissolve in media and remains in a crystal form; however, quercetin nanoparticles were well dissolved in media in both cell lines.

Quercetin is also well known for its anticancer activity (Reyes-Farias and Carrasco-Pozo 2019). To identify the anti-proliferation activity of QN, I performed the MTS assay in HCT-116 and U2OS cell lines following treatment of FQ dissolved in DMSO/media or QN dissolved in media for 72 h (Fig. 16A). Compared with each control, FQ dissolved in DMSO was most effective in inhibiting cancer cell proliferation, followed by QN dissolved in media.

I also performed the MTS assay in CCD-986sk fibroblast cells after treatment of FQ dissolved in DMSO/media and QN dissolved in media up to 72 h (Fig. 16B). Compared with each control, FQ dissolved in DMSO is most effective in inhibiting fibroblast cell proliferation and FQ dissolved in media did not have a significant effect since FQ cannot well dissolve in media. In the case of QN, the result confirms that QN in media increased proliferation compared to control. Based on these results, I can conclude that QN specifically and solely affects cancer cells.

To determine whether QN induces apoptosis of cancer cells, annexin V assay was performed and the percentage of apoptotic cells was measured after treatment of FQ, SSPS with chitosan and QN dissolved in media for 24 h (Fig. 17). There is evidence reported that quercetin can activate both intrinsic and extrinsic pathways of apoptosis (Rather and Bhagat 2020). When SSPS with chitosan was treated into the HCT-116 cells (Fig. 17, left bottom), early apoptotic cells were at 2.40% and late apoptotic cells were at 0.26%. whereas, QN treatment (Fig. 17, right bottom) increased both early and late apoptotic cells at 28.4% and 1.17%, respectively. This indicating that the QN treatment effectively induces apoptosis. QN treatment showed notably higher apoptotic cells compared to FQ treatment, due to the higher enhancing dissolved ability of QN in the cell culture media.

To identify additional mechanisms related to anticancer activity, I measured the

protein expression levels of NAG-1, p53 and SP1 after treatment of FQ and QN dissolved in media for 24 h (Fig. 18). In both treatments, pro-apoptotic proteins, NAG-1 and p53, were increased compared to control, while, and anti-apoptotic protein SP1 was decreased. Overall, quercetin nanoparticles exhibit similar or better anticancer effects compared to free quercetin.

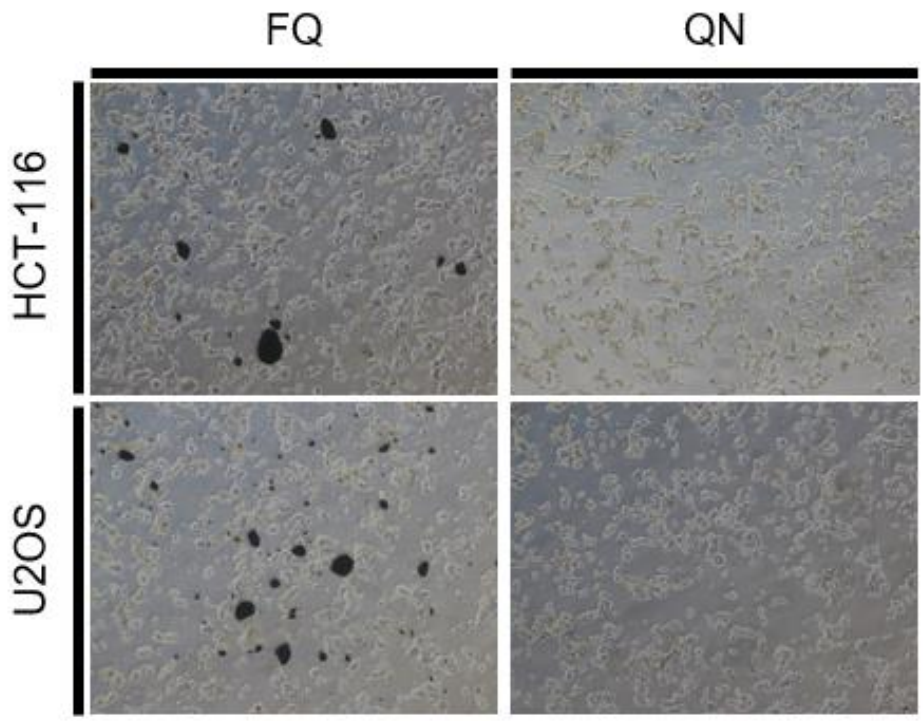

Figure 15. Image of HCT-116 and U2OS cells treated with FQ and QN dissolved in media.

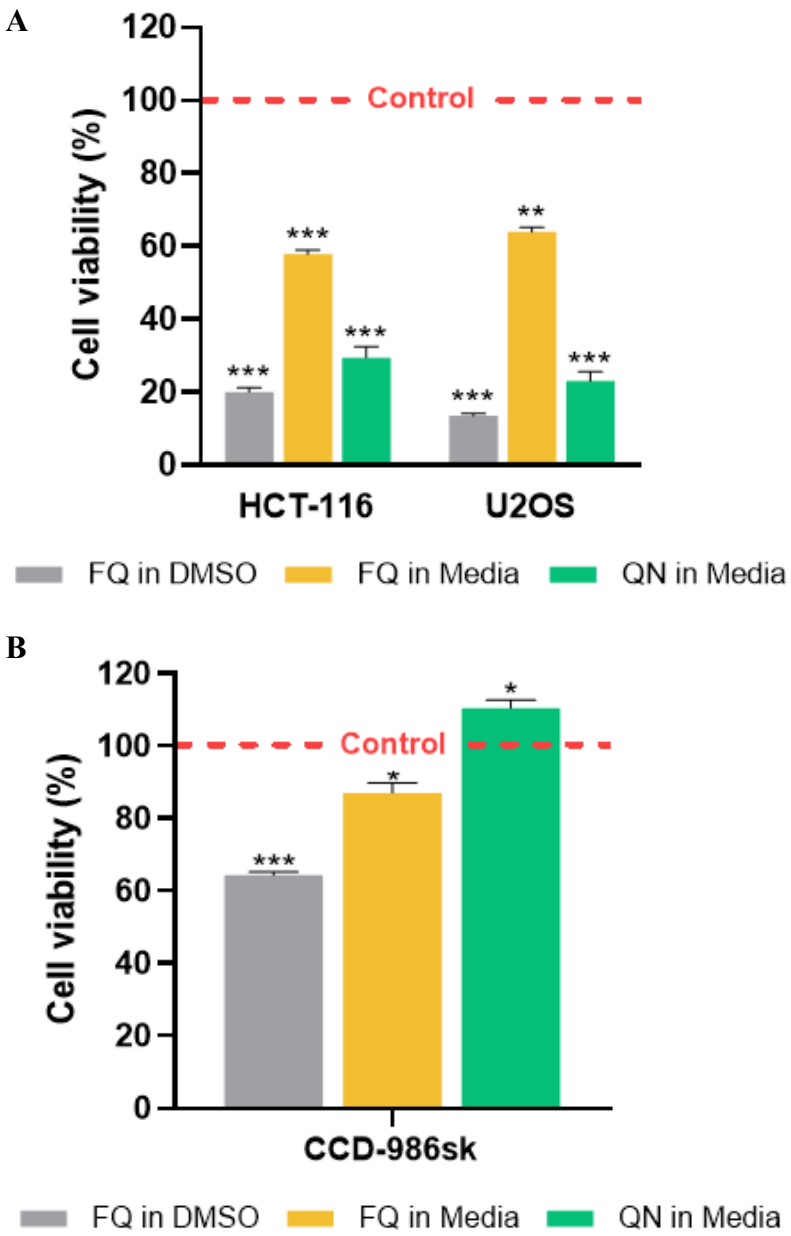

Figure 16. Cell viability of HCT-116, U2OS, CCD-986sk cells treated with FQ dissolved in DMSO and media, QN dissolved in media for 72 h. DMSO treatment used as the control for FQ dissolved in DMSO, media only treated cells used as the control for FQ in media, and SSPS with chitosan dissolved in media used as the control for QN dissolved in media. Each bar graph represents relative cell viability compared to each control. Data are shown as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control.

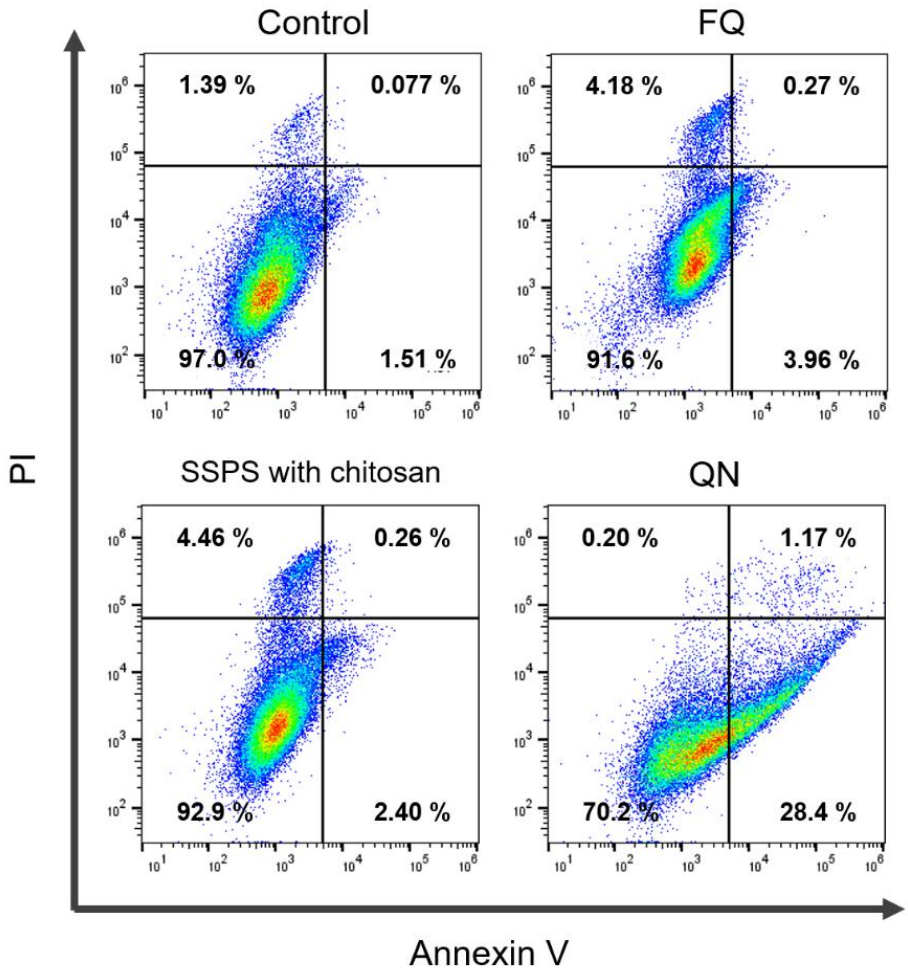

Figure 17. Apoptosis assay by annexin V assay kit using HCT-116 cells treated with FQ, SSPS with chitosan, and QN dissolved in media for 24 h. The right windows represent the population of apoptotic cells. PI, propidium Iodide.

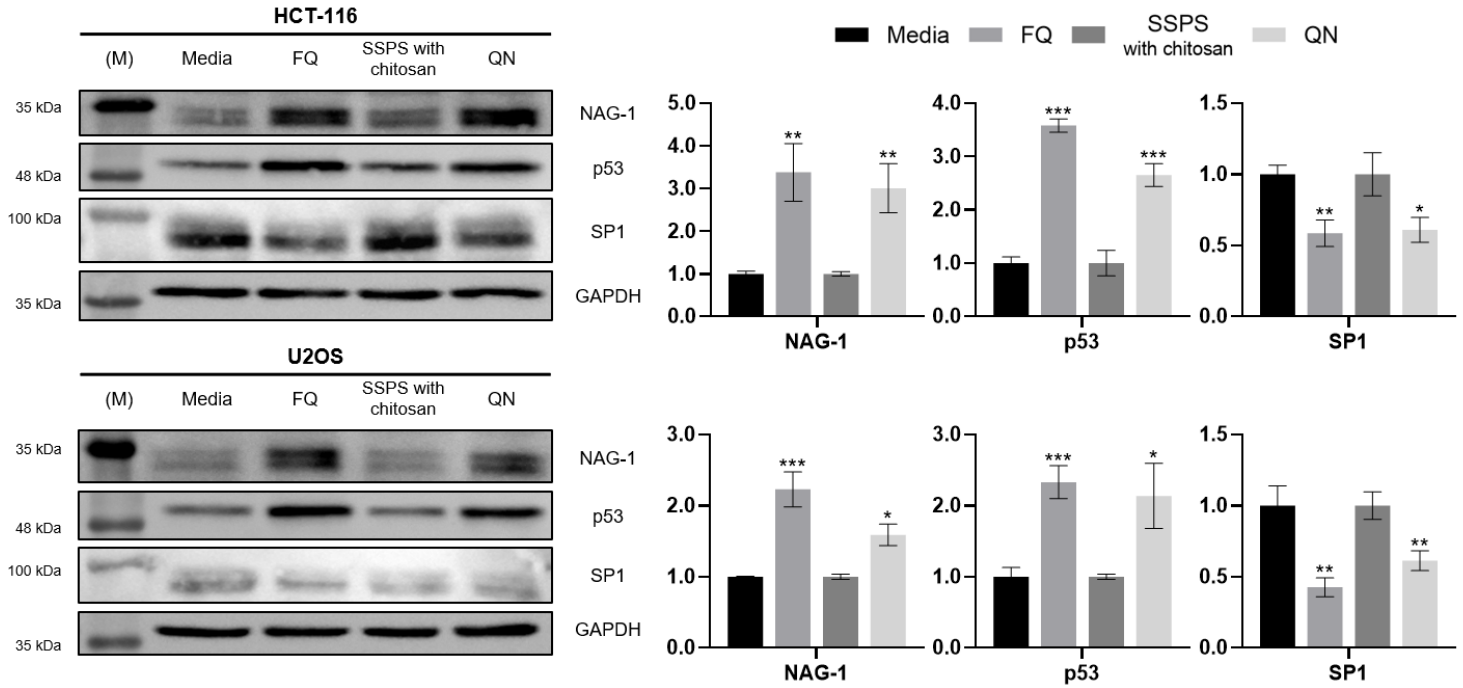

Figure 18. Western blot analysis of HCT-116 and U2OS cells treated with FQ dissolved in media, QN dissolved in media for 24 h. All experiments used quercetin concentration as 50 µg/ml. The media only treated cells used as the control for FQ in media, and SSPS with chitosan dissolved in media used as the control for QN dissolved in media. M indicates size marker. The data represent from three independent experiments. Data are shown as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control.

4. Discussion

Quercetin is a flavonoid found in daily foods including nuts, teas, vegetables, and numerous plants. Quercetin is believed to exert many beneficial effects on the health, including protection against various diseases such as cancer, allergy, and cardiovascular diseases (Ulusoy and Sanlier 2020). Recently, quercetin has been linked to the expression of pro NAG-1, thereby enhancing its anticancer activity in several cancers (Hong et al. 2021). However, the utilization of quercetin is imitated by its poor bioavailability, poor absorption, rapid metabolism, chemical instability, and rapid systemic elimination (Cai et al. 2013).

Nanoparticles (NPs) offer a promising platform to enhance quercetin bioavailability and various nano-carriers for quercetin delivery have been reported using natural biopolymers (Baksi et al. 2018), synthetic polymers (Halder et al. 2018), polymeric micelles (Chen et al. 2016), metallic NPs (Sadhukhan et al. 2019), and lipid-based NPs (Niazvand et al. 2019). These quercetin NPs including the one reported in this manuscript demonstrated enhancing the solubility and stability of quercetin is resulting in improving, cellular absorption, and reducing toxicity by protecting it from premature degradation in the body (Salehi et al. 2020). These results may provide the possibility for further studies using quercetin with nanotechnology-related strategies, in synergistic combination with existing clinically approved drugs.

Polysaccharides are natural polymers often used as safe and low-cost food ingredients that have attracted attention for the delivery of bioactive food components (Fathi, Martín, and McClements 2014). Polysaccharides have been incorporated into NPs and their activity has been investigated for many years (Seidi et al. 2018). Polysaccharide-based NPs exhibit several advantages compared to other NPs. First, using a natural biomaterial like polysaccharides to prepare NPs as opposed to metals or other synthetic polymers, minimizes concerns over cytotoxicity, biodegradability, and physiological stability. Secondly, polysaccharides are abundant in nature, making them cost-effective biomaterials. Moreover, studies show that polysaccharide-based NPs may decrease uptake by the mononuclear phagocyte system in comparison with other types of NPs (Kumari, Yadav, and

Yadav 2010), which prolongs the NP *in vivo* stability and increases the possibility of disease site accumulation. The residence time of polysaccharide-based materials is also extended due to the adhesion of polysaccharides, particularly to mucosal surfaces (Miao et al. 2018). These advantages allow for the development of potential therapeutic tools with bioactive ingredients including phytochemicals.

Among polysaccharides, soluble soybean polysaccharide (SSPS) is an acidic water-soluble polysaccharide consisting mainly of dietary fiber of soybean cotyledon and can isolate from okara, which is a by-product generated during tofu or soymilk production processes (Maeda and Nakamura 2021). SSPS has strong adhesive property, anti-oxidative property, excellent film-forming ability, and emulsifying property, which make them use in functional food ingredients. SSPS has been used in acidic beverages since it possess the property of dispersing and stabilizing milk protein in acidic conditions at a pH lower than the isoelectric point of milk protein (Nakamura et al. 2003). Most notably, SSPS has been demonstrated as an effective nanocarrier to encapsulate curcumin (Pan et al. 2018). Therefore, SSPS exhibits many potentials in NPs for essential components. At a near neutral pH, SSPS is negatively charged following interaction with the polycation to form polyelectrolyte complexes. In addition, these NPs are held together by molecular entanglement, electrostatic interactions, hydrogen bonding, and hydrophobic interactions (Li et al. 2018).

Chitosan is a natural polysaccharide that is positively charged because its amino groups are partially or completely dissociated in acidic solutions. As a nanocarrier, chitosan has been studied extensively due to its advantages as a natural polymer such as biocompatibility, biodegradability, non-toxicity, and wide distribution with low cost. Chitosan is rich in hydroxyl (-OH) and amine (-NH) functional groups, which can be used to react with crosslinkers for in situ chemical crosslinking. Historically, chitosan nanoparticles have been one of the most extensively studied natural biopolymer materials for biomedical and food applications.

In this study, I successfully encapsulated the quercetin in SSPS NPs and enhanced its stability by adding chitosan. The pH-dependent solubility properties of quercetin and its ability to absorb the lipophilic molecules of SSPS lead to possibly develop biopolymer NPs, however, but the stability could not be maintained for a long period. Yet, the addition of a small amount of chitosan, which has an opposite

charge to SSPS, increased the stability of SSPS based nanoparticles. This outcome was assessed and confirmed with several physicochemical analyses. Over the last decade, it has been demonstrated that hydrophobically modified polysaccharide derivatives can self-assemble into spherical nanoparticles with sizes in the range of 50 to 500 nm (Gericke, Schulze, and Heinze 2020). The size of QN in this study ranged from 10-100 nm with an approximate mean size of 24 nm, which is a relatively small spherical nanoparticle size thereby suggesting that it is highly stable. Furthermore, DSC and FTIR analysis showed quercetin was well encapsulated in the polysaccharides and this was confirmed by the disappearance of the peak indicating specific characteristics of quercetin.

The potent anti-oxidant activity, which play positive roles in various disease including cancer, is the well-known property of quercetin. To ensure this effect, we performed the ABTS and DPPH scavenging activity assay of each sample and luciferase reporter assay of ARE-promotor, which is the origin of NRF2 transcription that produces diverse anti-oxidant genes. The results of both assay indicated that QN and FQ have comparable anti-oxidant property. Furthermore, I investigated the other important biological activity of QN compared to free quercetin including anti-inflammation and anticancer effects. These experiments were confirmed that the anti-oxidant, anti-inflammation, and anticancer activities of the QN were not significantly different from the free quercetin.

5. **Conclusion**

Based on my results, I believe QN could potentially replace water-insoluble free quercetin while also maintaining its function. The QN could also be added to a solution thereby producing a drink that is supplemented with bioactive and soluble quercetin.

Polysaccharide-based nanoparticles can have multiple biomedical applications. They can be used alone and in combination with a target specific drug for the treatment of diseases. Furthermore, these nanoparticles can be used in personalized medicine to tackle unmet clinical needs that are highly variable in patients. Thus, there should be a considerable focus on the medical translation of nanoparticles, which means significant work is required in nanoscale manufacturing, scale-up, and regulatory requirements.

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국문 초록

다당류에 의해 나노캡슐화 된 퀘르세틴(Quercetin)을 통한 항암, 항염 그리고 항산화 활성의 개선 효과

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퀘르세틴(Quercetin)을 포함한 식이 폴리페놀 성분은 식물의 2 차 대사산물로서 암, 당뇨와 같은 다양한 만성 질환 예방에 도움을 준다. 그러나 퀘르세틴을 포함한 대부분의 플라보노이드계 화합물들은 열과 산화조건에 대한 불안정성과 불수용성에 대한 문제점이 지속적으로 제기되어 왔다. 또한, 경구 흡수성 및 생체 이용률이 낮아 단일물질로서 퀘르세틴을 체내 치료목적으로 활용하는 것은 한계가 있다.

수용성대두다당류(Soluble soybean polysaccharide) 및 키토산(Chitosan)과 같은 다당류 계열 바이오 폴리머 물질들은 나노입자 (Nanoparticle) 재료로써 다양한 분야에서 연구되고 있다. 나노입자는 기능성 물질 또는 약물을 실어 우리 체내의 다양한 장벽

(예컨대, 혈액뇌장벽 (blood-brain barrier) 을 통과하여 원하는 곳으로 전달할 수 있도록 돕는다. 이러한 장점은 퀘르세틴과 같은 소수성 플라보노이드 계열 화합물의 가장 큰 단점인 체내 흡수율을 극복해 줄 수 있는 기술로 주목받고 있다.

따라서, 본 연구는 수용성 다당류와 키토산을 수용체로 이용하여 퀘르세틴의 나노입자 및 체내 전달 시스템을 개발하고, 세포 내 항산화, 항염증 그리고 항암 효과를 확인하고자 하였다. 그 결과는 다음과 같다.

pH 사이클 조절을 이용해 수용성대두다당류에 퀘르세틴을 나노캡슐화 하였고, 추가로 키토산을 더해줌으로써 나노입자의 안정성을 증가시켰다. 위와 같이 제조한 수용성대두다당류와 키토산 기반의 퀘르세틴 나노입자의 이화학적 특성을 DLS, 제타(ζ) 전위, FT-IR, DSC 그리고 TEM 실험을 통해 분석하였고, 그 결과 퀘르세틴이 성공적으로 나노캡슐화 되었다는 것을 확인하였다. 또한 western blot, luciferase assay, cell proliferation assay 등 여러 세포 기반 실험을 통해 퀘르세틴 나노입자가 퀘르세틴 단일 입자와 비교하여 더 나은 항산화, 항염 그리고 항암 활성을 나타내는 것을 증명하였다.

따라서 본 연구 결과는 pH 를 조절하는 간단한 방법을 이용한 다당류 기반 나노 입자 제조 방법을 제시함으로써 퀘르세틴과 같은 플라보노이드 계열 화합물의 단점이었던 낮은 생체 이용률과 비수용성을 극복하고, 향후 생물의학 및 식품 산업에서 더 나은 활용 가능성을 기여할 수 있다고 여겨진다.

주요어: 생체고분자 나노입자; 자가 조립; pH 기반 방법; 수용성대두다당류; 키토산; 퀘르세틴; 항산화; 항암; 항염

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