Fabrication and characteristics of new quaternized chitosan nanocapsules loaded with thymol or thyme essential oil as effective SARS-CoV-2 inhibitors

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Contents:

1- Materials and instrumentation

2- Synthesis

1. Materials and instrumentation

Chemicals were obtained from the following suppliers and used without further purification: $AgNO_3$, epoxychloropropane, *N*,*N*-Dimethyldecylamine (DMDA)**,** and *N*,*N*-dimethylhexadecylamine (DMHDA) (Sigma–Aldrich).

Elemental analyses for C, H, N, were performed with a Perkin–Elmer 263 elemental analyzer. FT-IR spectra were recorded on a BRUKER Tensor-37 FT-IR spectrophotometer in the range 400–4000 cm^{-1} as KBr disc in the 4000-550 cm^{-1} region with 2 cm^{-1} resolution or with an ATR (attenuated total reflection) unit (Platinum ATR-QL, diamond). For signal intensities the following abbreviations were used: br (broad), sh (sharp), w (weak), m (medium), s (strong), vs (very strong). UV/Vis spectra were measured at 25 °C in ethanol (10^{-5} mol/L) on a Shimadzu UV-2450 spectrophotometer using quartz cuvettes (1 cm). NMR-spectra were obtained with a Bruker Avance DRX200 (200 MHz for ^1H) or Bruker Avance DRX500 spectrometer with calibration to the residual proton solvent signal in DMSO-d_6 (^1H NMR: 2.52 ppm, ^{13}C NMR: 39.5 ppm), $CDCl_3$ (^1H NMR: 7.26 ppm) against TMS (δ = 0.00 ppm). Multiplicities of the signals were specified s (singlet), d (doublet), t (triplet), q (quartet) or m (multiplet). X-ray powder diffraction of the samples was obtained using a powder Rigaku X-ray diffractometer (PXRD; Rigaku, Japan). The scanning was done in the region of 2θ from 30 to 80 at 0.041/min with a time constant of 2 s. Zetapotential analysis was prepared with a constant concentration 1% (w/v). Three measurements were made for each sample, and the mean was reported as the zeta-potential value of the sample.

2. GC-MS Analysis

The chemical composition of the extracted TEO was investigated by the gas chromatography-mass spectrometry (GC-MS) technique using a TRACE GC Ultra Gas Chromatograph (THERMO Scientific Corp., USA) and a thermo MS detector (ISQ Single Quadrupole MS). A capillary column TR-5 MS (30 m × 0.32 mm ID, 0.25 μm film thickness) was used to fractionate the oil samples. Operating conditions were as follows: Split ratio, 1:10; He gas flow 1.0 mL/min; Injection volume 1.0 μL; Column temperature maintained at 40 °C for 1 min, then raised at 4 °C/min to 180 °C and held for 1 min, then raised at 4 °C/min to 240 °C at 1 °C/min; Injector, transfer line, and detector temperatures were 210 °C, 270 °C, and 210 °C, respectively. The isolated fractions were diluted with hexane (1:10 hexane, v/v), and then 1 μL of the mixtures were injected, and mass spectra were obtained by electron ionization (EI) at 70 eV using a spectral range of m/z 40−450. To identify the chemical ingredients of the TEO, the chromatogram peaks were first de-convoluted with AMDIS software (www.amdis.net, accessed on 16 October 2021) and identified by matching the mass spectral patterns and retention indices of these ingredients to those of standard compounds or by comparing their mass spectra to those in the Wiley spectral library collection and NSIT library database [28]. In addition, the Kováts indices were calculated and compared to published retention indices. Compounds were quantified based on

their area in the chromatogram.

3. Synthesis

3.1. Extraction of β-chitin was the squid pens

3.1.1. Raw material

The raw material for the extraction of β-chitin was the squid pens from L. forbesi, which were kindly supplied by Fish and Seafood markets – Egypt. The pens were collected and then stored at −20 °C until being processed.

3.1.2. β-Chitin extraction

The squid pens were thoroughly washed with water, dried overnight at 30 °C, and then ground using a ball mill. Thereafter, sieving was performed to obtain the powdered pens fraction with average particle diameters in the range of 0.30–0.43 mm which was employed for the extraction of β-chitin. The β-chitin was obtained by performing the deproteinization and demineralization processes, consecutively [1]. The demineralization was carried out by washing the pens' powder twice with 0.55 M hydrochloric acid (10 mL per 1 g powder) at room temperature (RT) for 2 h each time. It is followed by thoroughly washing with distilled water till neutralizing the washing effluent (pH = 7). Afterward, the deproteinization was performed through the alkaline treatment for demineralized pens powder using an aqueous NaOH solution (0.3 M, 20 mL/1 g) at 82.5 ± 2.5 °C. This treatment was twice repeated for 1 h each. The change in color of the deproteinization medium from yellow to colorless indicates the almost complete removal of proteins from the chitin sample. Then washings were carried out up to neutrality, followed by drying in a vacuum oven at 50 °C for 24 h.

3.2. Preparation of ultrasound-assisted deacetylated squid chitosan (UCS)

The squid β-chitin was ground in a ball mill and fractionated using sieves. The fraction with particles of an average diameter in the range of 0.125–0.250 mm was subjected to the ultrasound-assisted deacetylation (USAD) process [28]. The β-chitin powder was suspended in an aqueous NaOH solution (10:1, volume (mL)/mass (g)) and the suspension was conveyed to a double-walled cylindrical glass reactor of internal diameter of 3.5 cm and equipped with a circulating thermostat to control the reaction temperature. Then the chitin suspension was subjected to ultrasonic irradiation in a LUHS-A17 sonicator (ν = 20 kHz). Based on the previous studies [28], the following operating conditions were selected for carrying out an USAD process; (i) β-chitin/NaOH = 1/10 g mL−1, (ii) Suspension volume = 50 mL, (iii) Irradiation pulse (IP = 0.5 s), (iv) Irradiation surface intensity (I = 52.6 Wcm−2), (v) reaction temperature (60 ± 1 °C), (vi) Reaction time = 50 min. After completion of the reaction duration, the reaction was quickly quenched by cooling the suspension to - 5 °C and neutralized by the addition of dilute HCl to precipitate the USAD product (UCS1) which was collected by filtration and thoroughly washed with deionized water

and freeze-dried. Applying the USAD process to USC1 for a further two consecutive times resulted in obtaining UCS2 and UCS3, respectively.

3.3. Synthesis of N-benzylidene chitosan (CSB)

Initially, a homogeneous UCS solution was obtained by dissolving UCS3 (2.0 g, 11.67 mmol NH_2) in 100 mL of 2% aqueous acetic acid under stirring at ambient temperatures for 1 h, then diluting the solution with 100 mL of ethanol and stirring it for another 15 min. Afterward, an ethanolic solution of benzaldehyde (equivalent to the molar NH_2-content in UCS3/ 50 mL of EtOH) was gradually added to the LMCS solution under vigorous stirring at 60 °C over a period of 15 min. Then, the reaction mixture was stirred for 6 h at the same temperature. The pH of the obtained solution was adjusted to 7.0 with 1 mol/L NaOH solution to precipitate the desired product. The precipitate was filtered, washed several times with anhydrous ethanol, and finally dried at 50 °C for 24 h. CSB was obtained as a light yellow solid, Yield:89%.^{1}H NMR (300 MHz, CD3COOD) δ (ppm): 8.61 (s, 1H, -CH=N-), 7.82 (d, 3H, -C_6H_5), 7.50 (s, 2H, -C_6H_5), 5.4–3.4 (m, 9H, CS), 2.04 (s, 1H, Acetyl-).

3.4. Thymol isolation

A hot water method was used to extract thymol from thyme essential oil (TEO) [1]. Briefly, in test tube, 1 mL of essential oil was dispersed in 2 mL of distilled water and the pH mixture was adjust to 13 using 0.5 mL of 0.1 M NaOH was added. The content was mixed with a vortex shaker for 2 minutes and then boiled at 100 °C for 10 min. Subsequently, the test tube was affixed to an immobile surface in order to facilitate the formation of two distinct phases. The oily phase was collected and its pH was adjusted to 7 with 2 M HCl. After that, 2 mL n-hexane was added to this phase and shaken with a vortex to completely dissolve thymol in n-hexane. The hexane layer (hexane + thymol) was isolated, and its solvent was evaporated to obtain the pure thymol.

[1] Jannati, N., Gharachorloo, M., & Honarvar, M. (2021). Extraction of thymol compound from Thymus vulgaris L. oil. *Journal of Medicinal plants and By-product*, *10*(1), 81-84.

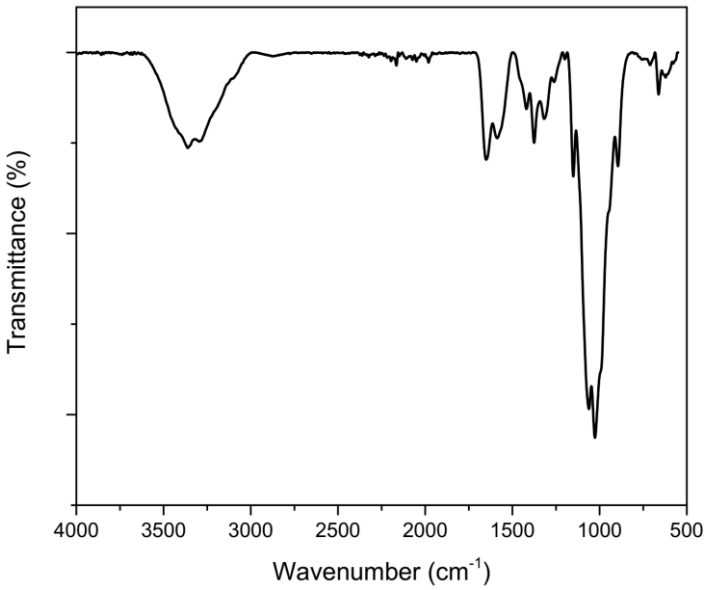

Fig S1: FTIR spectrum of QUCS

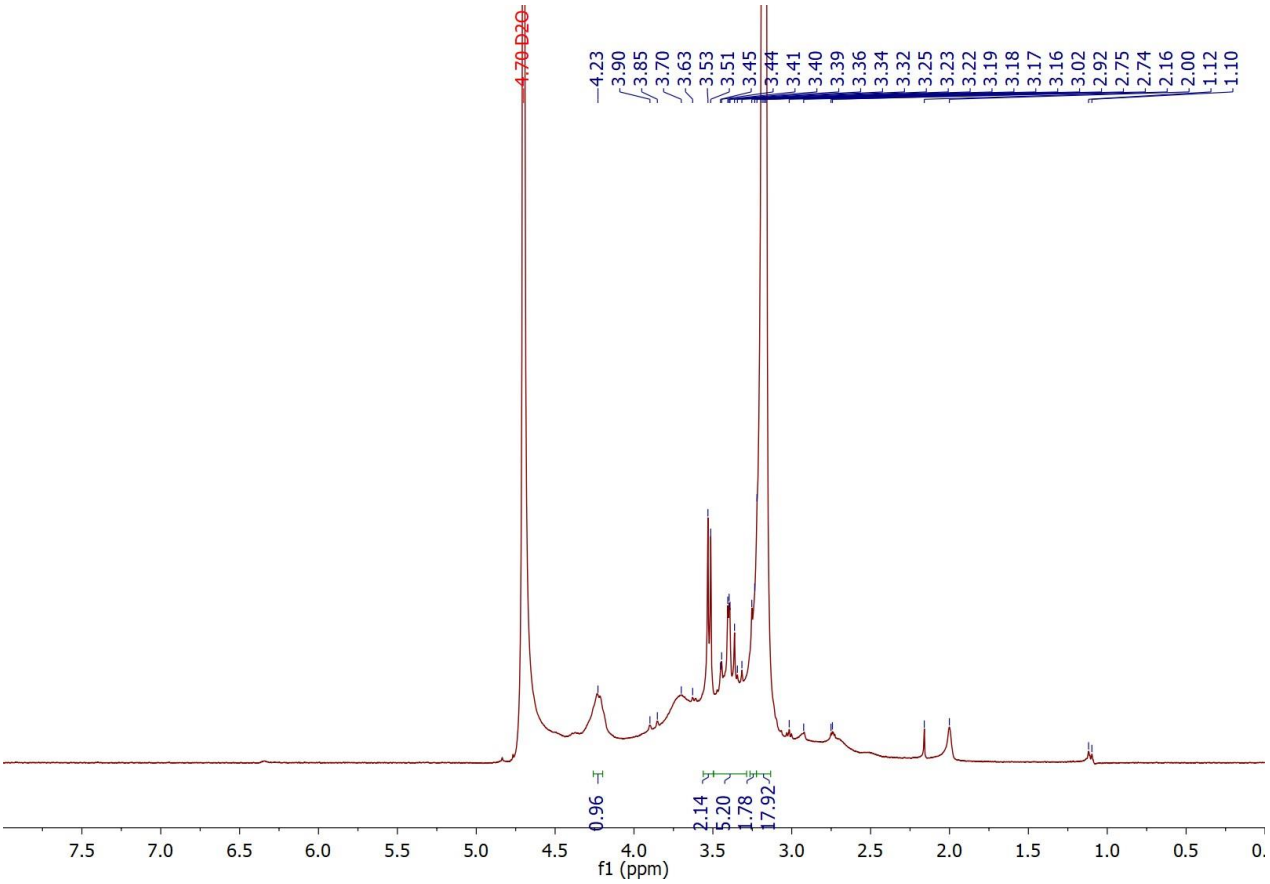

Fig S2: ^{1}H NMR spectrum of QUCS

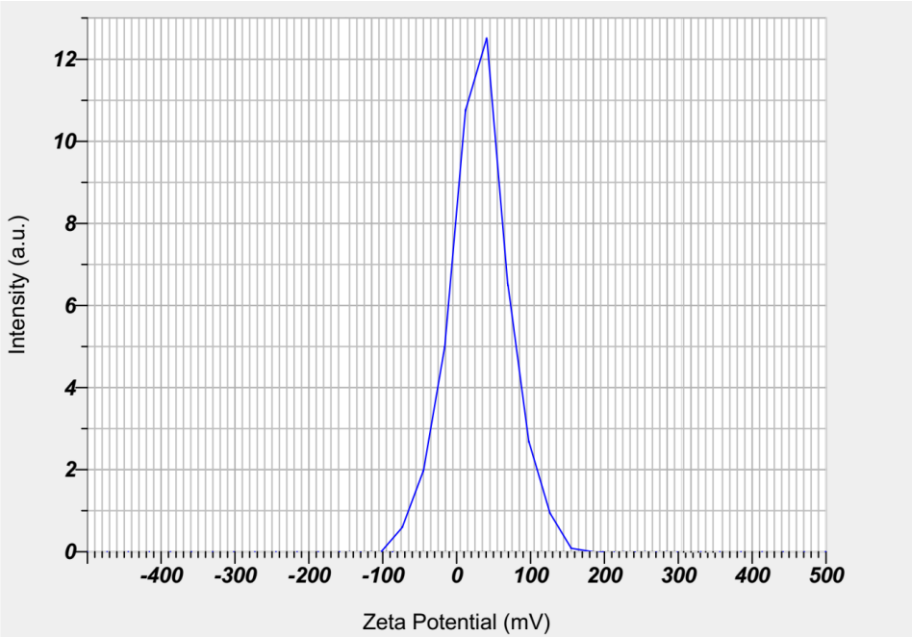

Fig S3: ZP (mV) of QUCS

References

[1] I. Kamal, A.I.M. Khedr, M.Y. Alfaifi, S.E.I. Elbehairi, R.F.M. Elshaarawy, A.S. Saad, Chemotherapeutic and chemopreventive potentials of ρ-coumaric acid – Squid chitosan nanogel loaded with Syzygium aromaticum essential oil, International Journal of Biological Macromolecules 188 (2021) 523-533.