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**Preparation of water-soluble chitosan via polyphenol conjugation and
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Abstract

Squid pen chitosan was modified by EGCG conjugation and carboxymethylation to improve water solubility, antioxidant activity, and antibacterial properties. Chitosan was prepared from squid pen chitin by alkaline deacetylation using 50% NaOH at 130 °C for 2 h. EGCG conjugates were prepared at 2-8% EGCG, while carboxymethyl chitosan (CMCS) was synthesized using monochloroacetic acid to molar ratios of 2:1, 4:1 and 6:1. Water solubility of CS-EGCG increased from 96% for CS-EGCG-2 to 98% for CS-EGCG-4 and 99% for both CS-EGCG-6 and CS-EGCG-8. CS-EGCG-8 showed the highest conjugation efficiency (91%), and antioxidant activity, with DPPH, ABTS, FRAP and Metal-chelating values of 106.33, 1045.92, 466.00 $\mu\text{mol TE/g}$ and 49.26 $\mu\text{mol EE/g}$, respectively. Within the CMCS series, water solubility increased from 82% for CMCS-2 to 89% for CMCS-4 and 98% for CMCS-6. CMCS-6 also showed the highest antioxidant and antibacterial activities within this series along with a degree of substitution of 0.20. FTIR showed a shift of the O-H/N-H band from 3451 to 3431 cm^{-1} after EGCG conjugation, whereas CMCS displayed carboxylate bands at 1601-1602 and 1421-1424 cm^{-1} . In ^1H NMR, CS-EGCG-8 showed signals at δ 6.0-7.0 ppm, while CMCS-6 showed additional resonances at δ 3.0-4.5 ppm. ^1H - ^{13}C HSQC further supported structural modification of both derivatives. Overall, EGCG conjugation provided the greatest enhancement in bioactivity, whereas carboxymethylation primarily improved aqueous solubility and selected functional properties.

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50 1. Introduction

51 Chitosan, a deacetylated derivative of chitin, is a versatile biopolymer widely
52 recognized for its biodegradability, biocompatibility, and intrinsic bioactivity. Because of
53 these properties, chitosan has been extensively investigated for applications in food
54 preservation, pharmaceuticals, and biomedical materials. From a food science perspective,
55 the development of chitosan-based materials is particularly relevant because food quality
56 is affected by both microbial deterioration and oxidative reactions [1]. These processes can
57 reduce shelf-life, cause undesirable changes in odor, flavor, color, and nutritional quality,
58 and increase food loss during storage. Therefore, biodegradable polymers with combined
59 antioxidant and antibacterial functions are increasingly being investigated as functional
60 ingredients, food-contact materials, and active packaging components. Among the
61 available raw materials, squid pen β -chitin has attracted increasing attention as a promising
62 source for chitosan production due to its low mineral content and less compact crystalline
63 structure compared with conventional α -chitin sources, which can facilitate its extraction
64 and subsequent chemical modification [2,3]. Squid pens are generated in considerable
65 quantities during squid processing and are considered as by-products. Their conversion
66 into chitosan provides a practical route for recovering value from seafood-processing waste
67 while supporting waste reduction, circular bioeconomy principles, and more sustainable
68 utilization of marine resources. Previous studies have demonstrated that squid pens can
69 be converted into chitin, chitosan and chitooligosaccharide with functional properties
70 relevant to food preservation and other food-related applications [4]. This valorization
71 approach is consistent with the increasing interest in transforming food-processing by-
72 products into value-added functional materials [3,5] Despite these advantages, native
73 chitosan has poor solubility at neutral pH and moderate antioxidant and antimicrobial

74 properties, which restrict its broader functional applications. To address these limitations,
75 chemical modification has been widely explored as an effective strategy to tailor the
76 physicochemical and biological properties of chitosan. Among these approaches, grafting
77 with natural polyphenols has emerged as a promising method for improving functional
78 performance [6,7]. Epigallocatechin gallate (EGCG), one of the major catechins in green
79 tea, is well known for its strong antioxidant and antimicrobial activities. The conjugation of
80 EGCG onto the chitosan backbone through free radical-mediated grafting has been
81 reported to enhance radical-scavenging ability, reducing power and antibacterial activity,
82 making EGCG-grafted chitosan a promising bioactive derivative for functional applications
83 [8,9]. These properties are also important for food applications because EGCG-chitosan
84 conjugates may offer improved functional compatibility for incorporation into aqueous food-
85 related systems, edible coatings, and active packaging matrices. However, the
86 effectiveness of such materials depends on the degree of EGCG incorporation, the
87 accessibility of phenolic groups and the interaction between the conjugate and the food or
88 lipid environment [10]. Therefore, controlling the extent of EGCG grafting is important
89 because modification level can influence both the structural characteristics and biological
90 functionality of the resulting conjugate. In parallel, carboxymethylation of chitosan produced
91 carboxymethyl chitosan (CMCS), whose physicochemical and functional characteristics are
92 influenced by degree and site of substitution [11]. The carboxyl and amino groups of CMCS
93 may additionally contribute to antioxidant activity and metal-ion interaction, which are
94 relevant to the inhibition of oxidation in lipid-containing foods[12]. In addition, chitosan
95 derivatives can serve as suitable hydrogel-forming materials, which are associated with
96 their hydration capacity, viscoelastic behavior, and ability to incorporate functional bioactive

compounds. Such properties are of particular interest for applications as active drug carrier [13].

Although modified chitosan derivatives have demonstrated promising antioxidant and antibacterial properties, direct comparisons of polyphenol grafting and carboxymethylation using the same squid pen derived chitosan as the parent material remain limited. Therefore, this study aimed to prepare chitosan from squid pen β -chitin and subsequently modify it through two distinct approaches: EGCG grafting and carboxymethylation. The modified chitosan was characterized using FTIR and $^1\text{H}/2\text{D}$ NMR and their antioxidant and antimicrobial activities to evaluate the most promising derivatives for future bioactive and hydrogel-based applications.

2. Materials and methods

2.1. Chemicals and materials

Squid pens were purchased from the Sea Wealth Frozen Food, Songkhla, Thailand. EGCG was procured from Xi'an Julong Bio-Tech Co., Ltd. (Xi'an, China). The chemicals used were also acquired from Sigma-Aldrich (St. Louis, MO, USA), while microbiological media were purchased from OxoidTM (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Preparation of chitosan from squid pen

The squid pens, measuring 14-16 cm in length, were thoroughly washed with tap water to remove water soluble impurities and subsequently dried in a hot-air oven at 60 °C for 24 h. After drying, the squid pens were manually cut into smaller pieces and further ground using a Panasonic blender (Model MX-898N; Selangor, Malaysia). The ground material was then sieved to obtain particles smaller than 1 cm. Squid pen have a low ash

content, the demineralization step was omitted. Chitin extraction was initiated with deproteinization using 1.0 M NaOH at 80 °C for 80 min. The conditions were selected from the preliminary study. The deproteinized chitin samples were washed and dried at 60 °C in hot-air oven.

Chitosan was prepared from chitin at 130 °C for 2 h as per our previously optimized conditions [3]. Chitin was slowly added into Duran flask containing 50% (w/v) NaOH and the final chitin/solution ratio was adjusted to 1:50 (w/v). After deacetylation process, chitosan was filtered and washed using distilled water until the pH reached neutral, followed by drying using a hot-air oven at 60 °C for 12 h. Dried samples were put in a plastic zip lock bag and stored in a desiccator. The obtained chitosan had degree of deacetylation of 95% and crystallinity index of 20% as measured by NMR and XRD, respectively as following the methods described by Lavertu et al. [14] and Sagheer et al. [15].

$$DDA \% = \left[\frac{(H1 - D)}{\left(H1 - D + H - \frac{Ac}{3} \right)} \right] \times 100$$

where H1-D is the integrated area of the anomeric proton of the deacetylated glucosamine unit and H-Ac is the integrated area of the methyl protons of the residual N-acetyl group.

$$Crl = \left[\frac{I_{110} - I_{am}}{I_{110}} \right] \times 100$$

where I_{am} & I_{110} are the intensity of amorphous diffraction at 16° and maximum intensity at 20°, respectively.

2.3. Preparation of chitosan-EGCG conjugate

Chitosan (1 g) was dissolved in 100 mL of 1% (v/v) acetic acid using a magnetic stirrer for 18-24 h. The pH of the resulting solution was then adjusted to 5.0 using 1 M NaOH in a conical flask. Thereafter, 2 mL of 0.5 M H₂O₂ containing 0.05 g ascorbic acid were added and the mixture was incubated at 40 °C for 60 min in an orbital shaker water bath (Memmert, D-91126, Schwabach, Germany). Thereafter, EGCG was introduced in the mixture at levels of 2, 4, 6, and 8% w/w of chitosan and incubated at 40 °C for 24 h. The resulting mixtures were dialyzed in dialysis bags (molecular weight cut-off 12-14 KDa) against twenty volumes of 0.1 M sodium acetate buffer (pH 5.0) at 4 °C for 12 h. The dialysis buffer was replaced twice to remove unbound EGCG. The dialyzed samples were subsequently freeze-dried (Scanvac Model Coolsafe 55, Lynge, Denmark). The chitosan-EGCG conjugates prepared with 2, 4, 6, and 8% EGCG were designated as CS-EGCG-2, CS-EGCG-4, CS-EGCG-6, and CS-EGCG-8, respectively.

2.3.1 Conjugation efficiency

Conjugation efficiency was determined via measuring total phenolic content using the Folin-Ciocalteu reagent (FCR) as described by Lei & Wang. [16] with some modifications. Briefly, conjugate samples were collected before and after dialysis and 0.1 mL of each sample was mixed with 0.75 mL of sodium carbonate solution (6% w/v). The mixture was incubated for 60 min at room temperature and absorbance was measured at 760 nm using a UV-visible spectrophotometer (Shimadzu, Model UV-1800, Kyoto, Japan). EGCG was used as the standard (0-100 µg/mL) and the result was expressed as mg EGCG equivalent/g sample. In the present study, conjugation efficiency was operationally estimated as the blank corrected retention of Folin-Ciocalteu reactive material after dialysis. The calculated value represents phenolic retention through dialysis on the stated sample

and the value presented as percentage. Conjugation efficiency was calculated by using the following equation:

$$\text{Conjugate efficiency (\%)} = \frac{TPC_{after\ dialysis\ sample} - TPC_{after\ dialysis\ control}}{TPC_{before\ dialysis\ sample} - TPC_{before\ dialysis\ control}} \times 100$$

2.4. Preparation of carboxymethyl chitosan (CMCS)

CMCS was synthesized via alkalization with slight modifications [11]. Chitosan was alkalized using 50% (w/v) NaOH and stirred overnight at room temperature. A solution of monochloroacetic acid (MCA) in isopropanol (1:1 ratio) was divided into five equal parts and added dropwise to the mixture at 10-minute intervals and the MCA amount was calculated based on the amount of chitosan (glucosamine unit present in chitosan) which was mainly used to make CMCS derivatives. Etherification was then performed under continuous stirring at room temperature for 10 h. The molar ratios of MCA to glucosamine units were set at 2:1, 4:1, and 6:1. After the reaction was completed, the product was precipitated using excess acetone (2-3 times the sample volume). This process was repeated three times, followed by centrifugation at 11,000 rpm for 10 min. The precipitate was then dialyzed against ultrapure water for 2 days. Water during dialysis was replaced in every 6 hours interval and then lyophilized for further analysis.

2.4.1. Degree of substitution of carboxymethyl chitosan

The degree of substitution (DS) of CMCS samples was determined by potentiometric titration according to Kong et al. [17], with slight modification. Accurately weighed CMCS-2, CMCS-4 and CMCS-6 (0.25 g) were dissolved in 25.00 mL of 0.1000 M HCL. NaCl. The solution was titrated under continuous stirring with 0.1000 M NaOH containing 0.1 M NaCl.

After addition of titrant, the solution was allowed to equilibrate, and the pH was recorded using a calibrated pH meter. A blank containing the same volume and concentration of HCl without CMCS was titrated in the same manner. The titration curve was constructed by plotting pH against the cumulative volume of NaOH and the inflection points were determined from the first-derivative curve.

V_0 represents the NaOH volume consumed in the blank titration, V_1 the volume consumed by excess HCL, V_2 the volume corresponding to completion of Carboxyl group (-COOH) neutralization, and V_3 the volume corresponding to neutralization of protonated amino groups (-NH₃⁺ and -NH₂⁺ CH₂COO⁻). DS was calculated using the equations proposed by Kong et al. [17].

$$DS = \frac{203C(V_2 - V_1)}{m(1 - w) - 80C(V_2 - V_1) + 22C(V_3 - V_0) + 42C(V_3 - V_2)}$$

Where C is the concentration of sodium hydroxide standard solution (mol/L). m is the weight of the sample (mg), and w is the water content of the sample (%). 203, 42 and 80 are the molar weight of chitin residue, acetyl and -CH₂COONa group, respectively and twenty-two is the molar weight difference between -CH₂COONa and -CH₂COOH.

2.5. Solubility

Water solubility of chitosan derivatives was determined gravimetrically based on the amount of insoluble residue as per the protocol of Huang et al. [18]. Briefly, 10 mg of each sample was dispersed in 1.0 mL of deionized water to obtain a concentration of 1% (w/v) in a pre-weighed 1.5 mL Eppendorf tube. The samples were thoroughly mixed using a vortex mixer for 4 h to promote dissolution and subsequently centrifuged at 10000 ×g for 5 min at 25 °C. After centrifugation, the supernatant containing the soluble fraction was

carefully removed, while the insoluble residue was retained in the tube. The tube containing the insoluble residue was dried in a hot air oven at 60 °C until constant weight. The tube was cooled to room temperature and weighed. The mass of the insoluble residue was calculated from the difference between the mass of the tube containing the dried residue and the initial mass of empty dried tube. Water solubility was calculated according to the following equation:

$$\text{Water solubility (\%)} = \frac{\{W_0 - (W_2 - W_1)\}}{W_0} \times 100$$

Where W_0 is the initial dry sample mass (mg), W_1 is the mass of the empty, dried centrifuge tube (mg), and W_2 is the mass of the centrifuge tube containing the dried insoluble residue (mg). Water solubility was expressed as a percentage.

2.6. Antioxidant activity

The antioxidant activities of chitosan, CS-EGCG conjugates, and CMCS were determined by DPPH-RSA, ABTS-RSA, FRAP, and MCA assays [6]. Stock solutions of all samples were prepared at 0.1% (w/v). Native chitosan was dissolved in 1% (w/v) acetic acid to ensure complete solubilization, whereas CS-EGCG and CMCS samples were dissolved in distilled water [11].

Samples were combined with an equivalent volume of 0.15 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) prepared in ethanol, followed by vortex mixing for 30 seconds. The resulting mixture underwent dark incubation at ambient temperature (25 °C) for 30 min before spectrophotometric measurement at 517 nm. Antioxidant capacity was quantified against a Trolox calibration curve (10-60 µM) and results were reported as micromoles of Trolox equivalents per gram of sample (µmol TE/g).

Firstly, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) working solution was freshly prepared by dissolving 7.4 mM ABTS with 2.6 mM potassium persulfate in equal proportions and allowing the mixture to stir protected from light for 24 h. For each measurement, 150 μ L of sample was combined with 2850 μ L of the prepared ABTS solution, kept in darkness for 45 min, and subsequently analyzed spectrophotometrically at 734 nm. Antioxidant activity was quantified using a Trolox reference standard (0–500 μ M) and expressed as μ mol TE/g sample.

MCA was assessed by introducing 20 μ L of 2 mM ferrous chloride and 40 μ L of 5 mM ferrozine to the sample, followed by a 20 min incubation period. Absorbance was recorded at 562 nm. Chelation activity was determined by comparison to an EDTA calibration curve (10–60 μ M) and reported as micromoles of EDTA equivalents per gram of sample (μ mol EE/g).

The FRAP working solution was prepared by combining 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (dissolved in 40 mM hydrochloric acid), and 20 mM ferric chloride hexahydrate in a 10:1:1 ratio, followed by warming to 37 °C for 30 minutes. Sample aliquots (150 μ L) were added to 2850 μ L of pre-warmed FRAP solution, protected from light, and held at room temperature for 30 minutes prior to measurement at 593 nm. Reducing power was quantified against a Trolox standard (0–400 μ M) and expressed as μ mol TE/g sample.

2.7. Antibacterial activity

The antibacterial activity of the samples was determined by measuring minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of samples against *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli*. Overnight bacterial cultures were grown in tryptic soy broth (TSB) and serially diluted to obtain a final

inoculum of 1×10^6 CFU/mL. Briefly, 100 μ L of bacterial suspension (final bacterial inoculum in each well approximately 5×10^5 CFU/mL) was added to each well of a sterile 96-well microplate, followed by 100 μ L of test sample prepared in sterile TSB to give final concentrations ranging from 100 to 0.196 mg/mL [19,20]. Acetic acid 1% (v/v) was used as a solvent control to distinguish the antibacterial effect of the solvent from the chitosan, while TSB containing wells without sample served as growth control. After incubation at 37 °C for 24 h, the MIC was defined as the lowest concentration showing no visible bacterial growth. For MBC determination, 10 μ L aliquots from wells showing no visible growth were spotted onto TSA plates and incubated for a further 24 h at 37 °C. The MBC was recorded as the lowest concentration at which no bacterial colonies were observed onto TSA plates and incubated for a further 24 h at **37 °C**. The MBC was recorded operationally defined as the lowest concentration at which no bacterial colonies were observed following subculture. Since 10 μ L (0.01 mL) of the well suspension was plated, the nominal detection limit of the subculture procedure was approximately 100 CFU/mL. No specific neutralization step was applied before subculture. Therefore, carryover of residual test material onto TSA could not be completely excluded.

2.8. Structural characterization of chitosan and its selected derivatives

2.8.1. FTIR

The structural characteristics of chitosan, CS-EGCG conjugates and CMCS were analyzed using Fourier-transform infrared spectroscopy (FTIR) with the potassium bromide (KBr) pellet technique. The procedure was adapted from the method used for squid-pen chitosan by Singh et al. [3] Before analysis, the samples and spectroscopic-grade KBr were dried to minimize interference from moisture. 1-2 mg of each powdered sample was

thoroughly mixed with approximately 100 mg of dried KBr. The mixture was finely ground and compressed into a transparent pellet using a hydraulic press. The FTIR spectra were recorded over the wavenumber range of 4000-400 cm^{-1} . A KBr pellet was used as background reference.

2.8.2. Nuclear magnetic resonance (NMR)

The samples prepared were analyzed by ^1H NMR and 2D NMR for structural changes during conjugation and carboxymethylation. ^1H NMR spectra of native chitosan and its selected derivatives were recorded using a Bruker AVANCE NEO 500 MHz NMR spectrometer with D_2O as the solvent. The operating frequency for ^1H NMR was 500 MHz. The chemical shifts were expressed in parts per million (ppm) and referenced to the residual HOD of D_2O .

A Bruker AVANCE NEO 600 MHz FT-NMR spectrometer equipped with a CP BBO CryoProbe at STREC was used for the 2D NMR analysis. The operating frequencies were 600 MHz for ^1H and 150 MHz for ^{13}C . ^1H - ^{13}C HSQC spectra were acquired for all three samples. Spectra were processed using Bruker Topspin and chemical shifts were expressed as δ (ppm). The correlations were examined to support assignment of the chitosan backbone, carboxymethyl substituents, and EGCG-derived aromatic groups.

2.9. Statistical analysis

Water-solubility, antioxidant assays, potentiometric determinations of DS and antibacterial assays were performed in triplicate, and results were expressed as mean $\pm$ standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). Differences among means were determined using Duncan's multiple range test at $p < 0.05$,

while paired data were compared using student's t-test. Statistical analyses were performed using SPSS software version 23.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Effect of EGCG at various concentrations on TPC, and conjugation efficiency of squid pen chitosan

The TPC of the CS-EGCG conjugates increased with increasing EGCG levels. TPC values of chitosan conjugated with EGCG at 2%, 4%, 6% and 8% were 18.55, 29.88, 59.73 and 79.96 mg/g sample, respectively. Before the dialysis, TPC values were noticed 19.25, 33.94, 66.95 and 86.22 mg/g sample for 2-8% of EGCG, respectively. This reduction in TPC after dialysis suggested the removal of free EGCG or related compounds and this trend was consistent with TPC values measured before and after dialysis. However, a slight change in the TPC was noticed (from 18.06 to 17.84 mg/g sample), when chitosan solution was dialyzed. This indicates that dialysis removed the non-retained phenolic fraction while a substantial proportion of EGCG remained associated with the chitosan matrix. Conjugation efficiency also increased from 59.66% to 91.14% (Table 1), while EGCG concentration increased from 2%-8%. Mittal et al. [6] observed an increase in conjugation efficiency of shrimp shell chitosan when the concentration of EGCG was increased. This increase was more likely associated with the increased availability of EGCG molecules for covalent and non-covalent interactions with the amino and hydroxyl groups of chitosan, resulting in higher grafting efficiency at elevated concentrations [10,22]. The higher retention of EGCG-derived phenolic material as increasing EGCG levels may be associated with greater availability of EGCG-derived species for interaction with or incorporation into chitosan matrix. In an ascorbic acid/ H₂O₂ redox system, phenolic grafting

onto chitosan has been proposed to proceed through a radical-mediated pathway. Liu et al. [23] demonstrated that the ascorbic acid/ H_2O_2 redox pair system generates ascorbate radicals capable of inducing carbon centered radicals on chitosan, thereby facilitating coupling with phenolic compounds. However, since the specific chemical linkage between EGCG and chitosan was not directly identified in the present study, a unique covalent reaction pathway cannot be assigned. Hydrogen bonding and other non-covalent associations may also contribute to the retention of EGCG -derived material within the modified chitosan matrix.

3.2. Effect of various MCA to glucosamine molar ratios on the DS of squid pen carboxymethyl chitosan

The degree of substitution (DS) is an important structural parameter of carboxymethyl chitosan (CMCS), because it represents the average number of carboxymethyl groups introduced per chitosan repeating unit. Increasing the MCA-to-glucosamine-unit molar ratio from 2:1 to 4:1 and 6:1 progressively increased the DS from 0.07 for CMCS-2 to 0.15 for CMCS-4 and 0.20 for CMCS-6, respectively (Table 1). This concentration-dependent increase indicated that greater availability of monochloroacetic acid promoted the incorporation of carboxymethyl groups at reactive sites along the chitosan backbone. Chitosan contains several potential sites for carboxymethylation, particularly the amino group at C2 and the hydroxyl groups at C3 and C6. Depending on the reaction conditions, N-carboxymethylated, O-carboxymethylated, or mixed N, O-carboxymethylated derivatives can therefore be formed [17]. The measured DS values describe the overall average extent of carboxymethyl substitution and do not, by themselves, distinguish the relative contributions of N- and O-carboxymethylation.

Similarly, de Abreu and Campana-Filho [24] noticed increased DS (0.52 to 1.44) with increasing chitosan: MCA molar ratios from 1:4.3 to 1:8.6 at room temperature for 3-10 h, which resulted in the formation of N, O-carboxymethyl chitosan. It has been noticed that the highly substituted products were obtained when a greater excess of MCA was used, whereas prolongation of the reaction time alone did not produce a proportional increase in DS [24]. Greater MCA availability can favor substitution, in addition reaction conditions also influenced the DS.

3.3. Solubility of chitosan and its derivatives

Native chitosan, with a degree of deacetylation of 95%, exhibited low water solubility (2%; Table 1). This poor aqueous solubility under neutral or near-neutral conditions and is associated with the strong intra and intermolecular interactions among its polymer chains [25]. Chitosan consists of hydroxyl and amino groups that participate in extensive hydrogen bonding, contributing to the formation of an ordered and compact structure [25]. Fan et al. [25] reported that reduced crystallinity and expansion of the crystal lattice facilitated the penetration of LiOH/urea aqueous solution into modified chitosan. EGCG modification of chitosan conjugation enhanced its solubility with increasing conjugation efficiency. Among all conjugates CS-EGCG-8 and CS-EGCG-6 showed the highest water solubility of 99%, CS-EGCG-4 (98%) and CS-EGCG-2 (96%). The high aqueous solubility results from the disruption of the highly ordered hydrogen-bonded structure of native chitosan after phenolic grafting. Furthermore, hydroxyl groups from the EGCG might have enhanced the interactions with water.

Within the CMCS series, the increase in DS from 0.07 to 0.20 was accompanied by an increase in water solubility from 82% to 98% (Table 1), suggesting that greater

carboxymethyl substitution contributed to improved aqueous solubility. Bukzem et al. [11] also noticed the nearly complete solubility (99.6%) of CMCS with a DS of 1.86 under their optimized conditions. Similarly, Thanakkasaranee et al.[26] reported that CMCS with higher DS exhibited greater polarity and higher water solubility (88.9% to 95.5%) owing to the greater abundance of polar groups, including -OH, -NH₂ and -COOH functionalities. Moreover, Chaiwong et al. [27] suggested that interactions between its carboxylic groups and water molecules through hydrogen bonding could provide another possible reason for enhanced solubility.

Overall, the two modification routes enhanced aqueous solubility through related but chemically distinct mechanisms. EGCG conjugation introduced bulky polyphenolic moieties bearing numerous hydroxyl groups while disrupting the original chitosan chain organization, whereas carboxymethylation introduced smaller polar and ionizable carboxymethyl functionalities that may reduce polymer chain ordering. The resulting increase in polymer–water interactions, combined with reduced polymer self-association, provides a reasonable structural explanation for the markedly higher solubility of both CS-EGCG and CMCS derivatives compared with native chitosan.

3.4. Antioxidant activities of chitosan and its derivatives

Antioxidant activities of chitosan, CS-EGCG, and CMCS are shown in Table 1. Within the CS-EGCG series, DPPH-RSA, ABTS-RSA and FRAP progressively increased with increasing EGCG level, in which CS-EGCG-8 showed the highest antioxidant activities than the remaining conjugates and native chitosan. CS-EGCG-8 also showed the maximum MCA within the CS-EGCG conjugates as well as other conjugates than the native chitosan, except for CS-EGCG-2, which had lower MCA ($p < 0.05$). Results suggested that

as the EGCG concentration increased from 2% to 8%, a greater number of phenolic groups were grafted onto the chitosan chain, resulting in a higher antioxidant potential, especially against the radicals. In general, EGCG possesses eight phenolic hydroxyl groups, which could serve as potent hydrogen donors capable of neutralizing free radicals through hydrogen atom transfer and single electron transfer mechanisms [28]. Xie et al. [29] found that gallic-acid (10.37 mg/mL) grafted on chitosan (10.10 mg/mL) exhibited higher DPPH-, ABTS- and superoxide-radical-scavenging activities, reducing power, MCA, lipid-peroxidation inhibition than the native chitosan. Cho et al. [30] also prepared gallic acid grafted chitosan at the various grafting ratios (0.1:1, 0.25:1, 0.5:1 and 1:1) and derivative with the highest gallic acid content (1:1 ratio had the highest gallic acid content, 118.92 mg GA/g conjugate) showed the strongest inhibition against DPPH-radical (92.26%) and hydrogen-peroxide (93.15%). In general, DPPH and ABTS primarily reflect radical-scavenging capacity, whereas FRAP measures ferric-ion reducing ability and MCA reflects metal-ion chelation [31]. The different magnitudes of increase among these assays therefore indicate an assay-dependent antioxidant response rather than a single mechanism. Notably, CS-EGCG-8 showed a particularly pronounced improvement in FRAP activity, increasing from 213.50 $\mu\text{mol TE/g}$ for native chitosan to 466.00 $\mu\text{mol TE/g}$. This represents an increase of approximately 118%, which was greater than the corresponding increases in DPPH-RSA and ABTS-RSA. The result suggests that EGCG-derived hydroxyl groups contributed strongly to the electron-donating and ferric-reducing properties of the conjugate [4,7,16]. The MCA results further indicate that EGCG conjugation enhanced the ability of the Chitosan matrix to interact with metal ions. MCA increased from 36.21 $\mu\text{mol EE/g}$ for native chitosan to 49.26 $\mu\text{mol EE/g}$ for CS-EGCG-8. Thus, the antioxidant enhancement associated with EGCG conjugation was not restricted

to direct radical scavenging but also involved improved metal-chelating functionality. Collectively, these mechanisms account for the superior and dose-dependent antioxidant performance observed in the CS-EGCG conjugates.

The CMCS derivatives also showed improved antioxidant activity compared with the native chitosan in several assays. CMCS-6 gave higher values than CS for DPPH-RSA), ABTS-RSA, and MCA, although its FRAP value remained lower than that native chitosan (Table 1). The antioxidant behavior of CMCS was governed by both the degree/level of carboxymethylation and the assay principle applied. In the DPPH assay, neither CMCS-2 nor CMCS-4 differed significantly from native chitosan, suggesting that a low to moderate substitution level was insufficient to enhance hydrogen-atom/electron donation to the lipophilic DPPH radical. In the ABTS assay, CMCS-2 remained comparable to native chitosan, whereas CMCS-4 and CMCS-6 exhibited significantly higher scavenging activity, indicating that the introduction of carboxymethyl groups improved reactivity towards the hydrophilic ABTS radical cation. A similar substitution-dependent trend was observed for metal-chelating activity, in which only CMCS-6 showed a significant increase relative to native chitosan, consistent with a higher density of carboxyl groups available for coordination with Fe^{2+} . Conversely, all CMCS derivatives displayed lower FRAP values than native chitosan, implying that carboxymethylation reduced the ferric-reducing capacity, most likely through the consumption or steric shielding of free amino groups that contribute to electron transfer. Collectively, these results demonstrate that carboxymethylation does not uniformly enhance antioxidant capacity rather, its effect is assay-dependent, reflecting differences in reaction mechanism, radical polarity, and the functional groups involved. Similarly, Zhao et al. [32] found that CMCS showed stronger

435 DPPH-radical scavenging than native chitosan from squid cartilage, while its ferrous-ion-
436 chelating activity reached 100% at 1.2 mg/mL compared with 22.34% for native chitosan.
437 The enhanced radical-scavenging activity of CMCS may be associated with the introduction
438 of hydrophilic carboxymethyl groups and enhanced aqueous solubility [33].
439 Carboxymethylation can disrupt the extensive intra- and intermolecular hydrogen bonding
440 present in native chitosan, thereby increasing hydration and potentially improving the
441 accessibility of functional groups to reactive species [34]. In addition, the carboxylate
442 groups introduced through carboxymethylation may provide additional sites for metal-ion
443 interaction, which may explain the particularly pronounced improvement in MCA.
444 Approximately 31% increase in MCA observed for CMCS-6 relative to native chitosan,
445 which suggested that carboxymethylation may enhance the preventive antioxidant function
446 of chitosan by increasing its capacity to sequester metal ions and thereby reduce their
447 participation in oxidation-promoting reactions [35,36]. In contrast, the FRAP value of
448 CMCS-6 (198.50 ± 3.54 $\mu\text{mol TE/g}$) was lower than that of native chitosan (213.50 ± 3.54
449 $\mu\text{mol TE/g}$). This difference may arise because FRAP reflects electron-donating capacity
450 through Fe^{3+} reduction, whereas DPPH/ABTS scavenging and metal chelation involve
451 different reaction mechanisms. Carboxymethyl substitution alters the availability and
452 electronic environment of the amino and hydroxyl groups of chitosan and introduces
453 carboxylate groups that favor hydration and metal-ion coordination but may not enhance
454 Fe^{3+} -reducing ability to the same extent. Thus, the lower FRAP of CMCS-6, despite its
455 higher DPPH-RSA, ABTS-RSA, and MCA, demonstrates that the antioxidant effect of
456 carboxymethylation is mechanism- and assay-dependent rather than a uniform
457 enhancement of all antioxidant properties. This interpretation is consistent with previous

studies showing that the reducing power of carboxymethyl chitosan varies with its substitution pattern and degree of substitution [37].

Comparison between CS-EGCG and CMCS further highlights the different contributions of EGCG conjugation and carboxymethylation. CS-EGCG conjugates exhibited higher DPPH-RSA, ABTS-RSA, and FRAP values than the CMCS samples, with CS-EGCG-8 showing the highest antioxidant activity among all chitosan derivatives. The markedly greater activity of CS-EGCG conjugates can be attributed primarily to the incorporation of EGCG-derived phenolic groups, which provide additional hydrogen- and electron-donating sites capable of participating in radical-scavenging and reducing reactions. Nevertheless, CMCS samples displayed higher antioxidant activity than native chitosan in DPPH-RSA, ABTS-RSA, and MCA, whereas FRAP value remained lower. These findings indicate that the two modification strategies enhanced antioxidant functionality through different structural mechanisms. EGCG conjugation introduced phenolic hydrogen- and electron-donating groups, whereas carboxymethylation altered polymer hydration, solubility, functional-group accessibility, charge characteristics, and the availability of carboxylate groups for metal-ion interactions [38].

Overall, the results demonstrate that both EGCG conjugation and carboxymethylation enhanced selected antioxidant properties of chitosan, although their effects were dependent on the type of chemical modification and antioxidant assay. EGCG conjugation produced the most pronounced overall enhancement, whereas CMCS showed improvement in radical-scavenging and metal-chelating activities compared with native chitosan.

3.5. Antibacterial activity

The antibacterial activity of CS, CS-EGCG conjugates and CMCS is shown in Table 2. A substitution-dependent improvement was evident across the CS-EGCG series. Native chitosan itself showed appreciable activity, with MIC/MBC values of 6.25/12.50, 3.12/6.25 and 6.25/6.25 mg/mL against *E. coli*, *L. monocytogenes* and *S. aureus*, respectively. These values were lower than, or equal to, those of CS-EGCG-2 (12.50/12.50 mg/mL against all three organisms) and CS-EGCG-4 (12.50/12.50, 3.12/12.50 and 12.50/12.50 mg/mL against *E. coli*, *L. monocytogenes* and *S. aureus*, respectively), indicating that a low degree of EGCG conjugation conferred little or no antibacterial advantage. A modest improvement was observed for CS-EGCG-6 (6.25/12.50, 1.25/12.50 and 6.25/12.50 mg/mL), which was largely comparable to native chitosan in terms of MIC. In contrast, the most pronounced improvement was obtained with CS-EGCG-8, which gave MIC/MBC values of 3.12/3.12, 0.19/3.12 and 1.56/3.12 mg/mL against *E. coli*, *L. monocytogenes* and *S. aureus*, respectively, suggesting that a sufficiently high level of phenolic substitution is required to enhance antibacterial efficacy. Similarly, Kim et al. [39] reported that chitosan grafted with caffeic acid, ferulic acid or sinapic acid showed lower MIC values (0.05-0.33 mg/mL) and MBC values (0.30-0.45 mg/mL) against *P. aeruginosa* and *L. monocytogenes* than unmodified chitosan, which showed MIC and MBC ranges of 0.12-1.08 and 0.17-1.84 mg/mL, respectively. Among the three microorganisms tested, *L. monocytogenes* exhibited the lowest MIC for CS-EGCG-8 (0.19 mg/mL), substantially lower than those observed for *E. coli* (3.12 mg/mL) and *S. aureus* (1.56 mg/mL). Differences in bacterial susceptibility may be related to variations in cell-envelope architecture, surface charge, membrane composition, and accessibility of cellular targets [40,41]. Chitosan is known to exhibit species-dependent antibacterial activity, with the response being influenced by

molecular weight, degree of deacetylation, concentration, pH, ionic strength, and bacterial characteristics [40,42]. The enhanced antibacterial activity of CS-EGCG can be explained by the complementary contributions of the chitosan backbone and EGCG moieties. Protonated amino groups in chitosan can interact electrostatically with negatively charged components of bacterial cell surfaces, leading to changes in membrane permeability, membrane depolarization, and disturbance of cellular homeostasis. Studies have shown that chitosan can permeabilize bacterial membranes and interfere with membrane-associated processes [40]. Following EGCG conjugation, the phenolic moieties may provide additional interactions with bacterial membranes and cellular proteins. EGCG has been reported to cause disruption of bacterial membrane integrity, leakage of intracellular components, and inhibition of essential cellular functions. Therefore, the increased activity of CS-EGCG may result from the combined action of the cationic polymer and the grafted polyphenolic groups [41,43]. These results indicate that increasing EGCG incorporation progressively increased the antibacterial potency of the conjugates, although the magnitude of improvement depended on the bacterial species. Free EGCG exhibited MIC/MBC values of 3.12/6.25 mg/mL against *E. coli*, 0.39/3.12 mg/mL against *L. monocytogenes*, and 3.12/6.25 mg/mL against *S. aureus*. CS-EGCG-8 was comparable or superior, with lower MBC values against *E. coli* and *S. aureus* and lower MIC values against *L. monocytogenes* and *S. aureus*. Conjugation therefore preserved the intrinsic activity of EGCG and, in some cases, enhanced it, consistently with a cooperative effect between the polycationic backbone and the grafted phenolic groups [41].

The CMCS derivatives showed weaker activity than CS-EGCG conjugate and native chitosan. However, with increasing DS, antibacterial activity was increased as suggested by lower MIC/MBC values (Table 2). CMCS-2 had MIC/MBC values of 50/50, 25/50 and

25/50 mg/mL against *E. coli*, *L. monocytogenes* and *S. aureus*, respectively, which was lower in CMCS-4 (25/50, 12.50/25 and 12.50/25 mg/mL). While CMCS-6 showed the greatest activity within the CMCS series, with MIC/MBC values of 12.50/12.50, 6.25/12.50 and 6.25/12.50 mg/mL, respectively. Thus, the higher MCA ratio was associated with improved antibacterial performance under the present synthesis conditions. Liu et al. [42]. reported that antibacterial activity against *E. coli* followed the order N, O-carboxymethyl chitosan < native chitosan < O-carboxymethyl chitosan, demonstrating that the antibacterial performance of carboxymethylated chitosan is strongly influenced by the site of substitution [63]. The lower antibacterial activity of CMCS relative to native chitosan may be related to changes in the cationic character of the polymer following carboxymethylation [42]. Antibacterial activity of chitosan is strongly associated with protonated amino groups that interact electrostatically with negatively charged bacterial cell surfaces [40]. Introduction of carboxymethyl groups can alter the charge density and accessibility of these amino groups, potentially weakening electrostatic interactions with bacterial membranes. At the same time, increasing MCA incorporation may modify polymer solubility, surface charge, and functional-group accessibility, which could explain the progressive improvement observed from 2:1 to 6:1 [42]. The present results therefore suggest that the antibacterial behavior of CMCS represents a balance between the structural effects of carboxymethylation and the extent of MCA incorporation.

Overall, CS-EGCG-8 was the most promising antibacterial derivative, whereas CMCS-6 was the most effective formulation within the CMCS series, although it did not surpass native chitosan.

3.6. FTIR analysis

FTIR spectra of native chitosan and its derivatives are shown in Figure 1a and 1b. The FTIR spectrum of native chitosan showed the characteristic broad band in the 3200-3500 cm^{-1} region, which is assigned to overlapping O-H and N-H stretching vibrations, together with absorption bands around 1600 cm^{-1} and 1000–1100 cm^{-1} , corresponding to characteristic amino/amide-associated vibrations and C–O/C–O–C stretching vibrations of the chitosan polysaccharide backbone, respectively [7]. More specifically, the band near 1640-1650 cm^{-1} is generally associated with amide I (C=O stretching of residual N-acetyl groups), whereas the band near 1560-1600 cm^{-1} is mainly associated with N-H bending or protonated amino groups. The carbohydrate fingerprint region contains C-O-C stretching near approximately 1150 cm^{-1} and C-O/pyranose-ring vibrations around 1030-1080 cm^{-1} . In the present study, the broad band of chitosan observed at approximately 3451 cm^{-1} , shifted slightly to approximately 3431 cm^{-1} in the CS-EGCG conjugates. This shift toward a lower wavenumber suggests an alteration in the hydrogen-bonding environment following EGCG incorporation, possibly due to interactions between the hydroxyl groups of EGCG and the hydroxyl and amino groups of chitosan [44]. Compared with native chitosan, the CS-EGCG spectra also exhibited more pronounced absorption in the 1500-1650 cm^{-1} region, with bands appearing around 1589-1641 cm^{-1} , as well as changes in the 1413-1431 cm^{-1} and 1016-1089 cm^{-1} regions. These changes are consistent with the introduction of EGCG-related structural contributions, including aromatic ring-associated vibrations and phenolic C-O stretching, and provide supporting evidence for modification of the chitosan structure following catechin grafting [20]. The spectral differences became more evident as the EGCG level increased from 2% to 8%, indicating a concentration-dependent structural

modification of chitosan. This interpretation agrees with previous reports showing the EGCG grafted chitosan displays altered FTIR profiles, particularly in the hydroxyl/amine stretching and fingerprint regions [7,44].

After carboxymethylation, prominent bands appeared in the regions of approximately 1601-1602 cm^{-1} and 1421-1424 cm^{-1} , which are assigned to the asymmetric and symmetric stretching vibrations of carboxylate groups ($-\text{COO}^-$), the region near 1600 cm^{-1} , because the region near 1600 cm^{-1} overlaps the native chitosan amino/amide bands and the region near 1420 cm^{-1} may overlap CH_2 bending vibrations respectively [11]. The increasingly evident carboxylate-associated features with increasing MCA: glucosamine ratio further suggests a greater extent of structural modification under the present synthesis conditions [45]. In addition, CMCS samples showed stronger absorption around 1000-1100 cm^{-1} region, with bands around 1070-1076 cm^{-1} . This is consistent with enhanced C-O or C-O-C and pyranose-ring vibration of the polysaccharide and introduced carboxymethyl groups after chemical substitution of the parent chitosan structure [46]. The broad band in the 3200-3500 cm^{-1} region was also retained after modification, indicating that O-H and N-H containing groups remained present in the CMCS structure, while changes in the band profile suggest alteration of the hydrogen-bonding environment following carboxymethylation. Among the CMCS derivatives, CMCS 6 showed the most pronounced carboxylate-associated spectral features, suggesting the greatest extent of structural modification under the synthesis conditions used. Nevertheless, peak intensity alone should not be used to quantitatively assign the degree of substitution unless supported by an independent quantitative method [11]. This interpretation is consistent with Bukzem et al. [11], who identified FTIR along with ^1H NMR as key evidence for carboxymethylation. Furthermore, recent studies show that CMCS derivatives are characterized by clear

carboxylate-related absorption bands in the $\sim 1600\text{ cm}^{-1}$ and $\sim 1420\text{ cm}^{-1}$ regions [11,47]. Collectively, these FTIR results support the successful chemical modification of native chitosan into EGCG-functionalized and carboxymethylated derivatives, although confirmation of the extent and covalent nature of modification should be considered together with complementary characterization data such as ^1H NMR and compositional analyses.

Based on the antioxidant assays and preliminary using the FTIR, CS-EGCG-8 and CMCS-6 were selected for further structural analysis.

3.7. ^1H NMR and 2D NMR analysis

The ^1H NMR spectrum of the selected CS-EGCG-8 retained the characteristic resonances of the chitosan backbone, including the overlapping ring-proton signals, predominantly H3-H6, in the $\delta \approx 3.6\text{-}4.0\text{ ppm}$ region, the H-2D signal near $\delta \approx 3.0\text{-}3.1\text{ ppm}$, and a signal at $\delta \approx 2.0\text{-}2.1\text{ ppm}$, tentatively assigned to N-acetyl methyl protons (H-Ac), because signals from residual acetate may also occur in the region, use of this resonance for quantitative DDA determination requires confirmation from the original integrated spectrum rather than peak position alone (Figure 2 a-c). The residual water signal (HOD) appeared at $\delta \approx 4.7\text{-}4.8\text{ ppm}$. The feature labelled H1-D at $\delta \approx 5.2\text{-}5.3\text{ ppm}$ was tentatively assigned to the anomeric proton of the deacetylated glucosamine unit. Because the HSQC anomeric correlation occurred in a different proton chemical shift region, the assignment of the $\delta \approx 5.2\text{-}5.3\text{ ppm}$ signal to H1-D should therefore be regarded as tentative. The spectrum of CS-EGCG-8 showed additional aromatic proton signals in the $\delta \approx 6.0\text{-}7.0\text{ ppm}$ region, which were not evident in native chitosan and can be attributed to the aromatic protons of EGCG-derived units [43]. The appearance of these new aromatic resonances, together

with changes in the polysaccharide proton region, supports the presence of EGCG-derived units in the modified material [43]. Similar spectral behavior has been reported for EGCG-grafted chitosan and other phenolic-conjugated chitosan systems, where emergence of aromatic proton signals is used as key evidence of successful conjugation [43]. This interpretation is also in line with the work of Mittal et al.[6], who reported that grafting EGCG onto chitosan produced conjugates with altered structural characteristics and improved functional performance compared with native chitosan.

The ^1H NMR spectrum of the CMCS-6 also showed characteristic chitosan-related signals (Figure 2c) including, the ring-proton region and the residual H-Ac signal at $\delta \approx 1.9$ -2.0 ppm. The HOD peak appeared at $\delta \approx 4.7$ –4.8 ppm. Compared with native chitosan, CMCS-6 exhibited additional spectral resonances in the expanded $\delta \approx 3.0$ -4.5 ppm region. These resonances may include contributions from carboxymethyl methylene protons. Because these signals overlap with the polysaccharide ring proton region, assignment to specific N- and O- carboxymethyl substitution sites could not be made unambiguously from present ^1H NMR spectra alone. These resonances may include contributions from the methylene protons of carboxymethyl substituents ($-\text{CH}_2 \text{COO}-$) in different substitution environments [24,48]. However, overlap with the polysaccharide ring-proton signals limits their unambiguous assignment to N- and O-substituted sites from the displayed ^1H NMR spectrum alone. The interpretation of these changes as evidence of carboxymethylation is consistent with Bukzem et al. [11], who confirmed carboxymethylation of chitosan using FTIR and ^1H NMR. The increased complexity of expanded region in the present spectrum therefore supports successful incorporation of carboxymethyl groups into the chitosan structure, while the coexistence of signals tentatively attributed to both N-site and O-site

substitution suggests that the selected derivative may contain mixed N, O-substitution. Confirmation of this substitution pattern requires complementary NMR evidence. Overall, the ^1H NMR result supports successful carboxymethylation to form CMCS-6. Its selection as the most suitable sample for subsequent evaluation should be supported by the combined structural and comparative functional results.

The ^1H - ^{13}C HSQC spectrum of native chitosan exhibited characteristic correlations attributable to the chitosan polysaccharide backbone (Figure. 3). The anomeric H1/C1 correlation was observed in the region of δ H 4.5-4.9/ δ C 95-105 ppm, while correlations corresponding to H2/C2 of glucosamine occurred around δ H 3.0-3.4/ δ C 50-60 ppm. Additional cross-peaks in the δ H 3.5-4.1/ δ C 70-83 ppm region were consistent with H3/C3, H4/C4, and H5/C5 correlations of the glucopyranose ring. H6/C6 correlations are expected at a lower carbon chemical shift, near δ C 60 ppm, and should be considered separately. These chemical-shift correlations are consistent with previously reported ^1H - ^{13}C HSQC assignments for chitosan [49]. The HSQC spectrum of CMCS-6 provided additional correlations tentatively attributed to the methylene groups of the introduced carboxymethyl substituents, supporting carboxymethylation of the chitosan backbone. These correlations are consistent with carboxymethyl substitution, although their assignment specifically to substitution at the C3 and C6 hydroxyl positions require resolved chemical-shift assignments and complementary connectivity evidence related O-carboxymethylated chitosan derivatives have shown carboxymethyl methylene correlations near δ C 71 ppm [48]. Such literature assignments provide a basis for comparison, but the precise chemical shifts and substitution sites in CMCS-6 should be established from its own NMR data. CS-EGCG conjugate exhibited characteristic cross-peaks corresponding to the chitosan glucosamine backbone. The correlation at δ H 4.9/ δ

~ 102 ppm was assigned to C1-H1, where cross peaks in δ H $\approx$ 3.0-4.0 ppm region consistent with overlapping chitosan-backbone correlations. Individual H2/C2-H6/C6 assignments were made only where the corresponding proton and carbon chemical shifts were sufficiently resolved; unresolved correlations were assigned to a specific glucosamine position. These assignments agree with previously reported NMR data for chitooligosaccharide. In addition, the expanded ^{1} H trace displayed resonances in the δ H $\approx$ 6.0-7.0 ppm region, consistent with EGCG-derived aromatic protons and supporting the corresponding observations in the ^{1} H NMR spectrum. Assignment of EGCG-associated HSQC cross-peaks requires confirmation of correlations between these proton resonances and carbons in the aromatic chemical-shift region. Similar structural features were reported for squid-pen chitooligosaccharide-EGCG conjugates, for which NMR analysis identified the characteristic chitosan carbon signals and an EGCG aromatic proton signal at approximately 7 ppm, together with changes in the C-2 and C-6 chemical environment after conjugation [43,44]. The HSQC results support retention of characteristic chitosan backbone correlations in the modified samples and provide complementary two-dimensional NMR evidence consistent with the FTIR and ^{1} H NMR findings. However, the HSQC displayed spectra alone does not establish the specific sites of carboxymethyl substitution or the covalent attachment of EGCG to chitosan.

4. Conclusion

Squid pen β -chitin was successfully converted into chitosan and subsequently modified through EGCG conjugation and carboxymethylation. Spectroscopic analyses supported structural modification of the chitosan backbone following both treatments. EGCG conjugation produced the most pronounced improvement in antioxidant and

antibacterial functionality, whereas carboxymethylation primarily enhanced aqueous solubility and selected antioxidant properties, particularly radical scavenging and metal chelation. Among the derivatives, CS-EGCG-8 showed the strongest overall bioactivity, while CMCS-6 was the most effective formulation within the carboxymethylated series. These findings support the potential of squid pen-derived chitosan as a value-added platform for developing multifunctional materials for food-related applications. Future work should focus on reproducibility, scalability, and establishing clearer structure–function relationships, particularly for the degree and site of substitution in CMCS.

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Declaration of Competing Interest

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Consent to Publish

Not applicable.

Consent to Participate

Not applicable

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT (OpenAI) to improve the clarity of the language. After using this tool/service, the author(s) reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Ethics statement

Ethics approval was not required for this research.

Clinical trial

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Data availability

All the data has been presented in the manuscript or supplementary files.

Author contribution

Rakesh Giri: Investigation, methodology, formal analysis, data curation, and writing—original draft. Soottawat Benjakul: Conceptualization, supervision, resources, and writing—review and editing. Mohammad Fikry: Formal analysis, validation, and writing—review and editing. Eng Keng Seow: Methodology, validation, and writing—review and editing. Avtar Singh: Conceptualization, methodology, supervision, project administration, and writing—review and editing. All authors read and approved the final manuscript.

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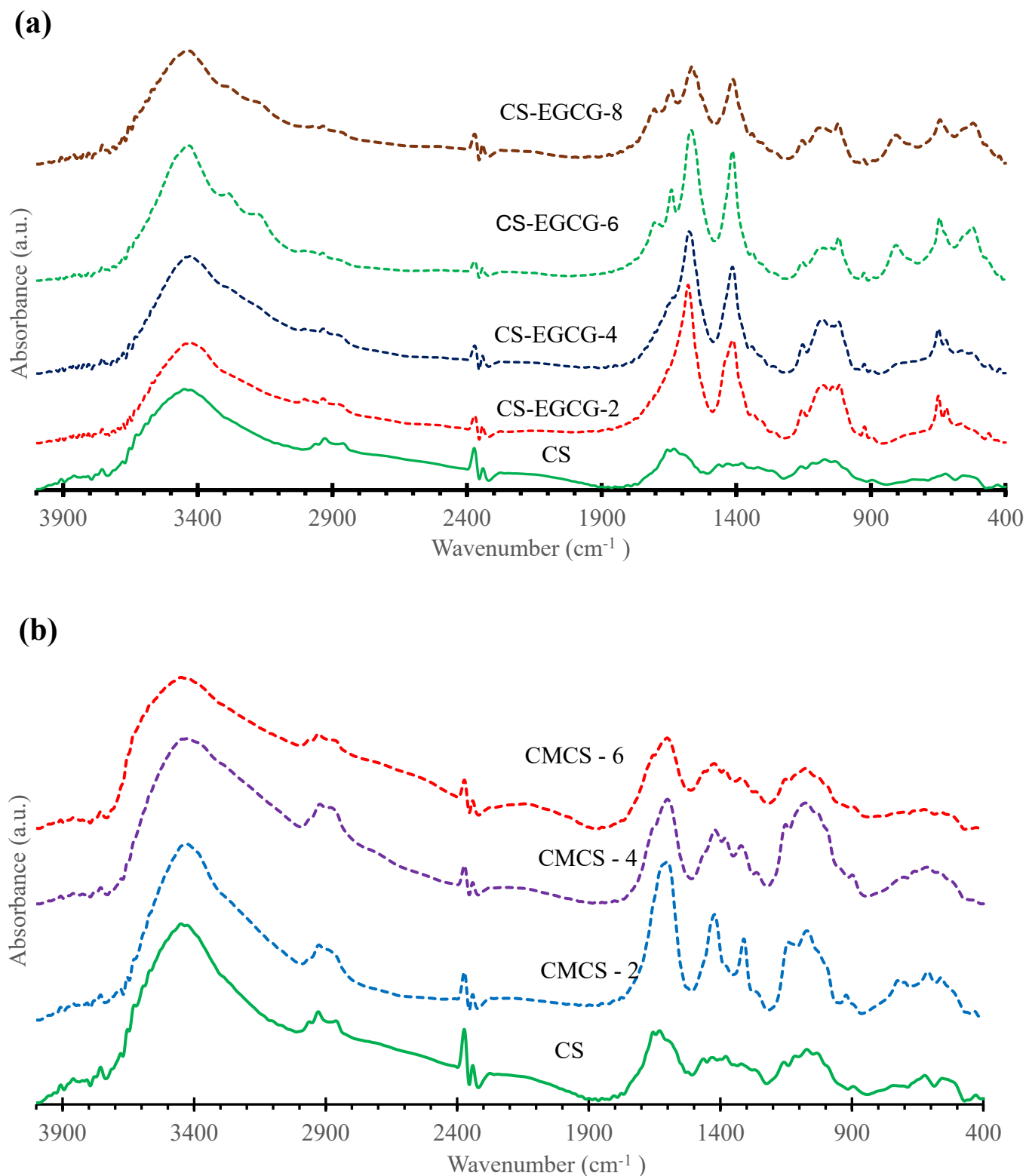


Fig. 1. FTIR spectra of squid pen chitosan and its derivatives prepared by (a) EGCG conjugation and (b) carboxymethylation. Panel (a) shows native chitosan (CS) and CS-EGCG conjugates prepared with 2%, 4%, 6% and 8% EGCG. Panel (b) shows native chitosan and carboxymethyl chitosan prepared at monochloroacetic acid to glucosamine ratios of 2:1 (CMCS 2), 4:1 (CMCS 4) and 6:1 (CMCS 6). The spectra were recorded using KBr-pellet method over 4000-400 cm⁻¹.

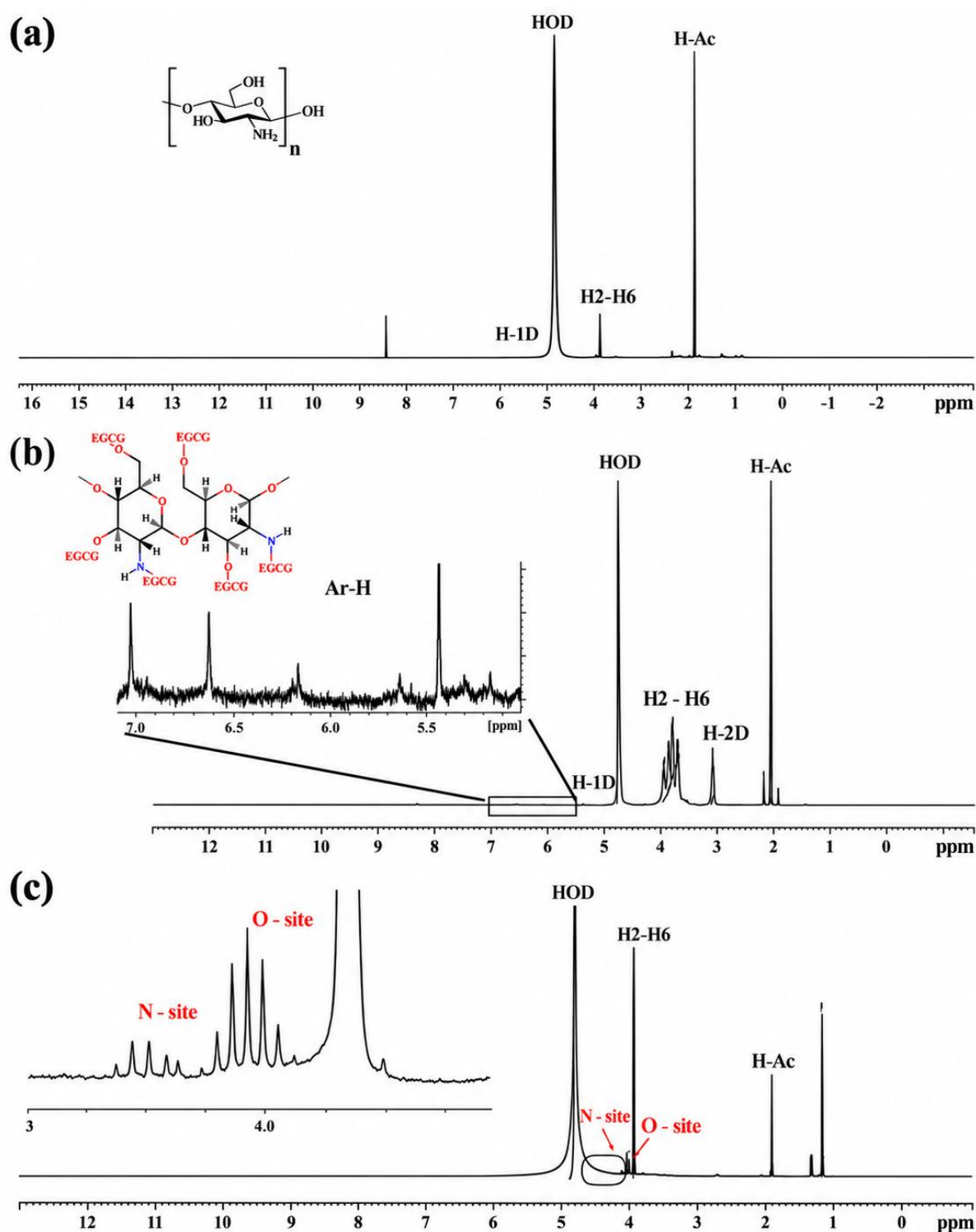
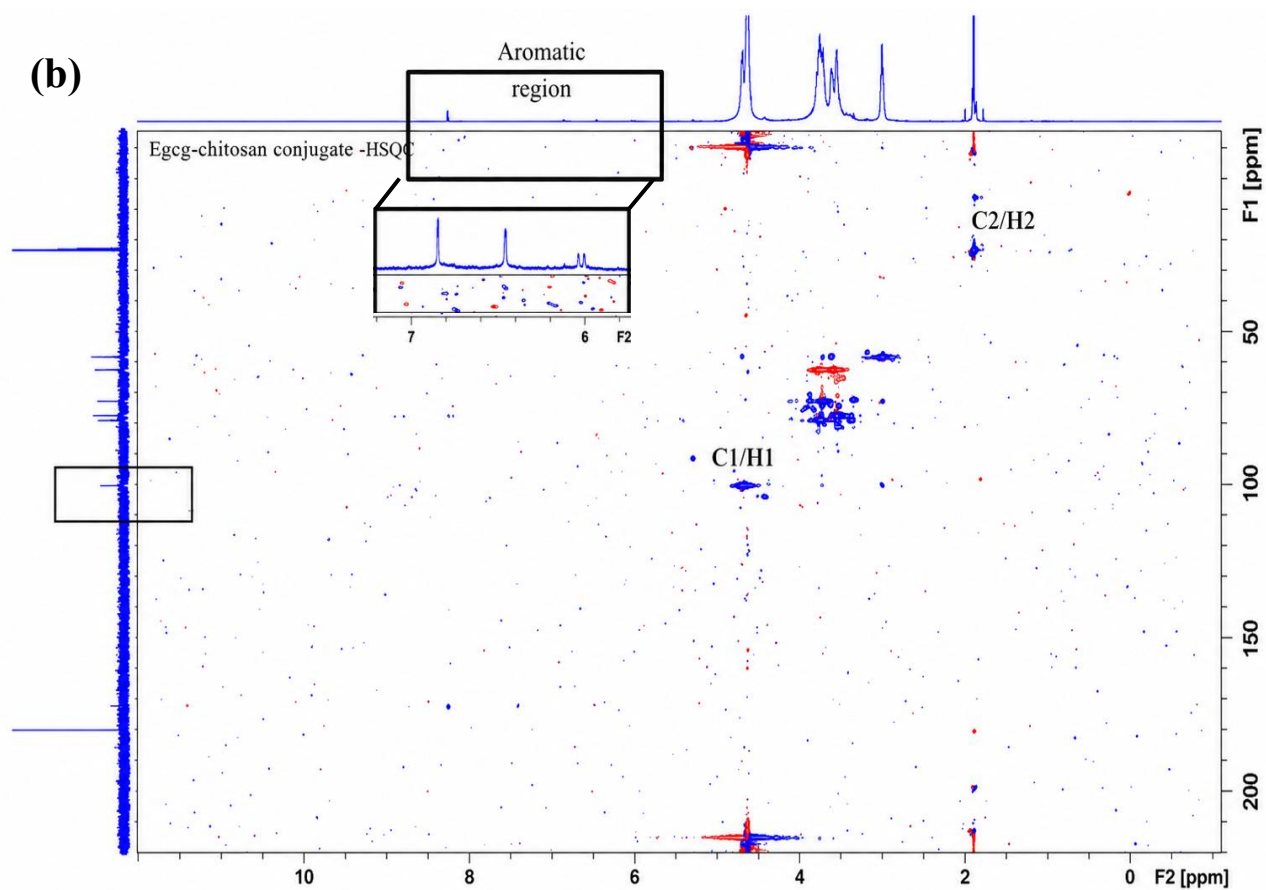
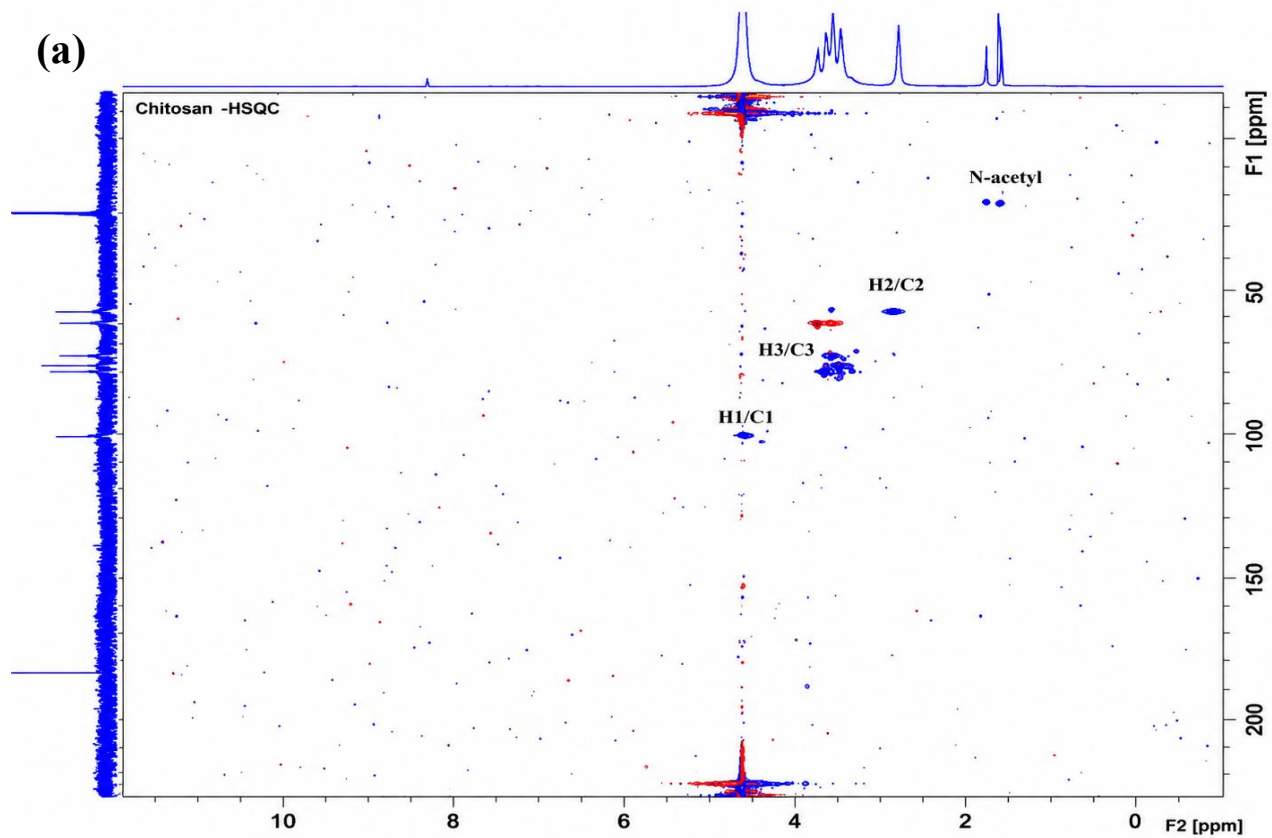


Fig. 2. ^1H -NMR spectra of chitosan (a) CS-EGCG-8 (b) and CMCS-6 (c) obtained from squid pen chitin. HOD, residual water proton; H-Ac, N-acetyl methyl protons; H1-D, anomeric proton of the deacetylated glucosamine unit; H2-D, proton attached to C2 of the deacetylated glucosamine unit; Ar-H, aromatic protons derived from EGCG. The N-site and o-site signal are tentative assignments. Chemical shifts are reported in ppm.



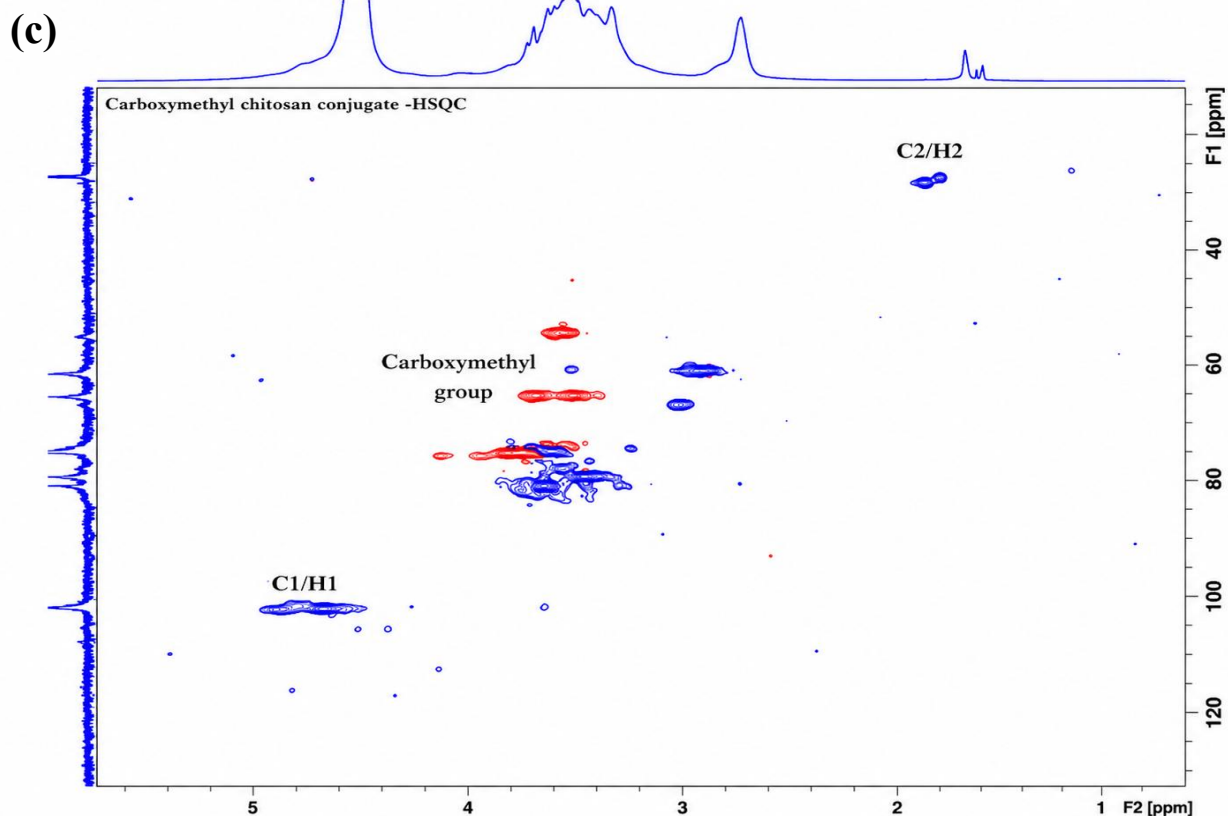


Fig. 3. 2D NMR spectra of chitosan (a) CS-EGCG-8 (b) and CMCS-6 (c) obtained from squid pen chitin. HSQC, heteronuclear single-quantum coherence; F1, ^{13}C chemical-shift dimension; F2, ^1H chemical shift dimension; H1/C1, H2/C2 and H3/C3, correlations from the chitosan glucopyranose backbone. Cross-peaks assigned to the aromatic region in CS-EGCG 8 and the carboxymethyl group in CMCS-6 are interpreted as supportive evidence of chemical modification. Spectra are shown as contour plots.

Table 1. Conjugation efficiency, degree of substitution, water solubility and antioxidant activities of chitosan and its various derivatives

Sample	Conjugation efficiency (CE;%)	Degree of substitution (DS)	Water solubility (%)	DPPH-RS-A ($\mu\text{mol TE/g}$)	ABTS-RS-A ($\mu\text{mol TE/g}$)	FRA-P ($\mu\text{mol TE/g}$)	MCA ($\mu\text{mol EE/g}$)
CS	-	-	2 $\pm$ 0.13	70.56 $\pm$ 3.45 ^f	684.67 $\pm$ 4.12 ^g	213.50 $\pm$ 3.54 ^f	36.21 $\pm$ 2.67 ^d
EGCG	-	-	100 $\pm$ 0.00	120.56 $\pm$ 0.57 ^a	1149.67 $\pm$ 6.48 ^a	3206.00 $\pm$ 7.07 ^a	80.24 $\pm$ 2.22 ^a
CS-EGCG-2	59.66	-	96 $\pm$ 0.13	77.07 $\pm$ 2.30 ^e	732.58 $\pm$ 1.18 ^f	253.50 $\pm$ 3.54 ^e	33.86 $\pm$ 0.13 ^d
CS-EGCG-4	75.82	-	98 $\pm$ 0.09	84.79 $\pm$ 1.72 ^d	867.17 $\pm$ 7.66 ^d	268.50 $\pm$ 10.61 ^d	38.51 $\pm$ 0.22 ^{bcd}
CS-EGCG-6	85.68	-	99 $\pm$ 0.04	97.39 $\pm$ 2.30 ^c	938.00 $\pm$ 8.84 ^c	316.00 $\pm$ 0.20 ^c	41.75 $\pm$ 0.18 ^{bcd}
CS-EGCG-8	91.14	-	99 $\pm$ 0.07	106.33 $\pm$ 1.15 ^b	1045.92 $\pm$ 1.18 ^b	466.00 $\pm$ 0.10 ^b	49.26 $\pm$ 3.16 ^b
CMCS-2	-	0.07	82 $\pm$ 0.25	67.72 $\pm$ 1.72 ^f	692.58 $\pm$ 4.71 ^g	193.50 $\pm$ 3.54 ^{gh}	36.62 $\pm$ 7.78 ^{cd}
CMCS-4	-	0.15	89 $\pm$ 1.30	71.78 $\pm$ 1.72 ^f	727.17 $\pm$ 6.48 ^f	186.00 $\pm$ 1.00 ^h	43.07 $\pm$ 8.72 ^{bcd}
CMCS-6	-	0.20	98 $\pm$ 0.34	77.47 $\pm$ 2.87 ^e	793.83 $\pm$ 0.59 ^e	198.50 $\pm$ 3.54 ^g	47.53 $\pm$ 4.54 ^{bc}

Values are expressed as mean $\pm$ standard deviation (SD) of triplicate determinations (n=3). Different superscript lowercase letters within the same column indicate significant differences among samples ($p < 0.05$). DPPH-RSA (2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity), ABTS-RSA (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity; FRAP (Ferric reducing antioxidant power); MCA (metal-chelating activity); TE (Trolox equivalent); EE (EDTA equivalent); CS (Chitosan); EGCG (Epigallocatechin gallate); CMCS (Carboxymethyl Chitosan).

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of chitosan and its various derivatives.

Samples	<i>Escherichia coli</i> (mg/ml)		<i>Listeria monocytogenes</i> (mg/ml)		<i>Staphylococcus aureus</i> (mg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC
Chitosan	6.25	12.50	3.12	6.25	6.25	6.25
Acetic acid	1.22	1.22	0.15	1.22	1.22	1.22
EGCG	3.12	6.25	0.39	3.12	3.12	6.25
CS-EGCG-2	12.50	12.50	12.50	12.50	12.50	12.50
CS-EGCG-4	12.50	12.50	3.12	12.50	12.50	12.50
CS-EGCG-6	6.25	12.50	1.25	12.50	6.25	12.5
CS-EGCG-8	3.12	3.12	0.19	3.12	1.56	3.12
CMCS-2	50.00	50.00	25.00	50.00	25.00	50.00
CMCS-4	25.00	50.00	12.50	25.00	12.50	25.00
CMCS-6	12.50	12.50	6.25	12.50	6.25	12.50

CS (chitosan); EGCG (epigallocatechin gallate); CMCS (carboxymethyl chitosan). Mic was defined as the lowest sample concentration showing no visible bacterial growth, whereas MBC was defined as the lowest sample concentration at which no bacterial colonies were detected after subculture