Chitosan-hydroxycinnamic acid conjugates: Optimization of the synthesis and investigation of the structure activity relationship

Vivien Nagy[a], Priyanka Sahariah[a], Martha Á. Hjálmarsdóttir[b], Már Másson[a*]

[a]Faculty of Pharmaceutical Sciences, School of Health Sciences, University of Iceland, Hofsvallagata 53, IS-107 Reykjavík, Iceland

[b]Faculty of Medicine, School of Health Sciences, University of Iceland, Hringbraut 31, IS-102 Reykjavík, Iceland

Abstract

A new synthesis method was developed and optimized by a full factorial design for conjugating hydroxycinnamic acids (HCA-s) to chitosan. Cinnamic acid and tertbutyldimethylsilyl protected HCA-s were converted to their corresponding acyl chlorides and reacted with tertbutyldimethylsilyl-chitosan to selectively form amide linkages, resulting in water-soluble conjugates after deprotection. Nineteen conjugates were obtained with various degrees of substitution (DS) ranging from 3% to 60%. The conjugates were found to be bactericidal against *Staphylococcus aureus* and *Escherichia coli*, with their activities equal to chitosan at low DS but an increase in the DS correlated with reduced activity. DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging assay was performed to determine the EC_{50} values. Chitosan only exhibited low antioxidant activity, whereas the HCA-chitosan conjugates exhibited higher antioxidant activities correlating with the DS. One caffeic acid conjugate (21%) was 4000 times more active than chitosan and more active than free caffeic acid.

Keywords

Chitosan, antioxidant, antibacterial, chemical modification, protection groups, Design of Experiment (DOE)

*Corresponding author. Tel: +354 525 4463; Fax: +354 525 4071
E-mail address: mmasson@hi.is (M. Masson)

1. Introduction

Chitosan is a linear polysaccharide derived from chitin, consisting of *D*-glucosamine and *N*-acetyl glucosamine monomers. It is a non-toxic biopolymer that can be used in food products, nutraceuticals, cosmetics, pharmaceuticals, bone-tissue engineering, agriculture, waste-water treatment, and other applications (Bakshi, Selvakumar, Kadirvelu, & Kumar, 2020; Deepthi, Venkatesan, Kim, Bumgardner, & Jayakumar, 2016; Másson, 2021c). In addition, chitosan possesses significant antimicrobial activity against gram-negative (e.g. *Escherichia coli*, *Pseudomonas fluorescens*) and gram-positive (e.g. *Staphylococcus aureus*, *Listeria monocytogenes*) bacteria and fungi (e.g. *Botrytis cinerea*, *Drechstera sorokiana*) (Sahariah & Másson, 2017), which is the basis for many applications.

The glucosamine monomers of chitosan have one free amino group and two hydroxyl groups, which can react to scavenge free radicals, and thus, the polymer could also act as an antioxidant (Feng, Du, Li, Hu, & Kennedy, 2008). The antioxidant properties of chitosan and its potential applications (e.g. drug delivery systems to provide controlled release, material for tissue engineering, food packaging) are the topic of many published studies (Nagy & Másson, 2020; Ngo et al., 2015). Some researchers have reported significant antioxidant activity of chitosan and chitooligosaccharides (COS-s) (T. Sun, Zhou, Xie, & Mao, 2007), while others have found a lack of activity (Moreno-Vasquez et al., 2017).

The antioxidant activity of chitosan can be further improved by conjugation with natural antioxidants. Woranuch et al. reported that chitosan had 41.4% 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging ability that could be increased to 96.6% by grafting ferulic acid onto the polymer backbone. The activity was found to be independent of the degree of substitution (DS) for ferulic acid (Woranuch & Yoksan, 2013). Eom et al. studied eight different phenolic acid conjugated COS-s and reported that while native COS-s expressed weak antioxidant activity, the derivatives had improved activities. It was also reported that hydroxycinnamic acid-chitooligosaccharide (HCA-COS) conjugates had better antioxidant activity than hydroxybenzoic acid-COS conjugates (Eom, Senevirathne, & Kim, 2012). In a study by İlyasoğlu et al., caffeic acid conjugated chitosan retained comparable DPPH radical scavenging activity as caffeic acid itself, with more than 90% scavenging activity at 1 mg/mL concentration (İlyasoğlu, Nadzieja, & Guo, 2019). Pasanphan et al. reported that unmodified chitosan did not reduce DPPH at 1200

μM concentration, while a gallic acid conjugate scavenged 87.3% of the free radical at the same concentration (Pasanphan & Chirachanchai, 2008). In a study by Lee et al., phenolic acid conjugated chitosan derivatives expressed a 1.79-5.05-fold increase in DPPH radical inhibition compared to unmodified chitosan (Lee, Woo, Ahn, & Je, 2014).

The antimicrobial activity of antioxidant conjugates has also been studied, and it has been reported that conjugation of HCA-s to chitosan can improve water-solubility as well as antimicrobial activity compared to unmodified chitosan, allowing the conjugates to be used at neutral conditions (Kim et al., 2017; Singh, Dutta, Kumar, Kureel, & Rai, 2019). Sun et al. studied four gallic acid-chitosan conjugates and reported increased antimicrobial activity against *Escherichia coli* (*E.coli*), *Salmonella typhimurium* and *Bacillus subtilis*. The strongest activity was exhibited by the conjugate with the highest DS (X. Sun, Wang, Kadouh, & Zhou, 2014). Lee et al. synthesized caffeic acid-chitosan conjugates which had improved antioxidant abilities compared to chitosan as well as improved antimicrobial activity against two standard methicillin-resistant *Staphylococcus aureus* (MRSA) strains and three standard methicillin-susceptible *Staphylococcus aureus* (MSSA) strains, as well as eight foodborne pathogens (e.g. *Enterococcus faecalis, Salmonella typhimurium*) (Lee et al., 2014). In a study by Badawy et al. the antifungal activity of cinnamic acid-chitosan conjugates was investigated. Against *Botrytis cinerea*, the conjugates exhibited a 12-fold increase in activity compared to chitosan (Badawy et al., 2004).

Although chitosan-antioxidant conjugates have been studied for various applications (Nagy & Másson, 2020), the influence of the DS and structural variations on biological activities is not fully understood. In some of the previous studies, the structural characterization was limited to IR spectroscopy, and the DS values were not defined. In many cases, it is also unclear if the chemical or enzymatic procedures (Aljawish, Chevalot, Jasniewski, Scher, & Muniglia, 2015; Li, Guan, Zhu, Wu, & Sun, 2019) used to graft antioxidant moieties to the polymer were sufficiently selective only to give the targeted structures for the conjugates.

In the present study, we have sought to address these issues to improve the understanding of the structure-activity relationship. Thus, we aimed to conjugate cinnamic-, *p*-coumaric-, ferulic, and caffeic acid to chitosan at different DS. A clear correlation is expected between the DS and the antioxidant and antimicrobial activities of chitosan-antioxidant conjugates. The antioxidants used in this study are commonly included in food products and pharmaceutics (Sova & Saso, 2020).

They share the same core structure but vary in the number and position of hydroxyl and methoxy groups on the phenyl moiety. Previously published procedures for *N*-acylation of chitosan with HCA-s were investigated but found to be lacking in the conversion efficiency. Thus, the synthesis was done starting from 3,6-di-*O*-tert-butyldimethylsilyl-chitosan (TBDMS-protected chitosan). Our group has previously shown that this procedure provides a controlled and selective conversion of chitosan to 3,6-di-*O*-TBDMS-chitosan with up to 100% DS (Rathinam, Ólafsdóttir, Jónsdóttir, Hjálmarsdóttir, & Másson, 2020; Rúnarsson, Malainer, Holappa, Sigurdsson, & Másson, 2008; Sahariah, Árnadóttir, & Masson, 2016; Sahariah et al., 2020). The aromatic OH groups on the antioxidants were also TBDMS protected so that all protection groups could be removed using similar conditions in the final reaction step. The conjugates were characterized by ^{1}H-NMR to confirm the structure and determine the DS. The antioxidant activity was determined by DPPH scavenging assay, and the antibacterial activity of the conjugates was evaluated by determining the minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) for *S. aureus* and *E. coli*.

2. Experimental

2.1 Materials

Marine chitosan derived from shrimp shells (Degree of deacetylation 88%, MW=136 kDa*) was donated by Primex ehf. Cinnamic acid (97%), ferulic acid (97%), caffeic acid (98%), *p*-coumaric acid (98%), L-ascorbic acid (≥99%), tert-butyldimethylsilyl chloride (TBDMSCl, 97%), imidazole (puriss ≥99%), sodium chloride (NaCl, puriss, ≥99%), thionyl chloride ($SOCl_2$, ≥99%), triethylamine (TEA), cinnamoyl chloride (98%), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HoBt), dimethylsulfoxide (DMSO), methanesulfonic acid (puriss ≥99.5%), hydrochloric acid 37% (HCl), N,N-dimethylformamide (DMF), dichloromethane (DCM), acetonitrile (ACN), ethyl acetate (EtOAc), methanol (MeOH), ethanol (EtOH), sodium bicarbonate ($NaHCO_3$), sodium sulfate (Na_2SO_4) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Germany). Glacial acetic acid (puriss 100%) was obtained from Merck. Mueller Hinton broth and Mueller Hinton Agar were obtained from Oxoid Ltd. All other chemicals and reagents used in this study were of analytical

grade and commercially available.

*Previously measured by SEC-MALLs following a previously published procedure by Rathinam et al. (Rathinam, Solodova, Kristjánsdóttir, Hjálmarsdóttir, & Másson, 2020a)

2.2 Synthesis of the chitosan mesylate salt

The mesylate salt of chitosan was prepared following a previously published procedure method described by Benediktsdóttir et al. (Benediktsdóttir et al., 2011). Yield: 1.3 g (85%).

2.3 Synthesis of 3,6-di-O-TBDMS-chitosan

3,6-di-*O*-TBDMS-chitosan was prepared following a previously published procedure method described by Benediktsdóttir et al. (Benediktsdóttir et al., 2011). Yield: 1.7 g (83 %).

2.4 Synthesis of TBDMS-HCA acids

2.4.1 Synthesis of p-TBDMS-coumaric acid

The synthesis of TBDMS-protected *p*-coumaric acid (4-*O*-tert-butyldimethylsilylcoumaric acid) was carried out according to the method described by Matsuno et al. (Matsuno, Nagatsu, Ogihara, & Mizukami, 2001). Yield: 0.71 g (42%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.15 ($(CH_3)_2C$, s, 6H), 0.92 ($(CH_3)_3C$, s, 9H), 6.24 (H-1, d, 1H), 6.78 (H-4, H-5, d, 2H), 7.37 (H-3, H-6, d, 2H), 7.66 (H-2, d, 1H).

2.4.2 Synthesis of TBDMS-ferulic acid and di-TBDMS-caffeic acid

The synthesis of TBDMS-ferulic acid (4-*O*-tert-butyldimethylsilylferulic acid) and di-TBDMS caffeic acid (3,4-di-*O*-tert-butyldimethylsilylcaffeic acid) was carried out following the same procedure as for the synthesis of *p*-TBDMS-coumaric acid synthesis. The products were obtained in 66 and 61% yields, respectively.

TBDMS-ferulic acid: ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.18 ($(CH_3)_2C$, s, 6H), 1.00 ($(CH_3)_3C$, s, 9H), 3.85 (CH3, s, 3H) 6.31 (H-1, d, 1H), 6.86 (H-4, d, 1H), 7.05 (H-3, H-5, d, 2H), 7.71 (H-2, d, 1H)

di-TBDMS-caffeic acid: ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.22 ($(CH_3)_2C$, s, 12H), 0.99 ($(CH_3)_3C$, s, 18H), 6.23 (H-1, d, 1H), 6.83 (H-3, d, 1H), 7.03 (H-4 and H-5 merge, s, 2H), 7.64

(H-2, d, 1H).

2.5 Synthesis of antioxidant-chitosan conjugates

The cinnamic acid/TBDMS protected HCA-s (0.5 g, 1 eq) were refluxed with $SOCl_2$ (11 mL, 8 eq) in DCM for 5 hours then concentrated in vacuo. Then, the acyl chloride was reacted with 3,6-di-*O*-TBDMS-chitosan (1 eq) and TEA (2 eq) in DCM for 24 h. The obtained crudes were concentrated by evaporating the solvent in a rotavapor, then re-dissolving them in DCM and concentrated again, which was followed by extensive washing with water and ACN. The obtained product was dried in a vacuum oven at 40 °C for 8 h. The final step was the deprotection by stirring 1 g of material in 150 mL of 2M HCl solution in MeOH for 48 h, followed by ion exchange in a 10 %w/v NaCl solution and dialysis. The dialysis water (receptor phase) was changed eight times. The materials were then lyophilized. The final products were obtained in 52-64% yields.

Cinnamic acid conjugated chitosan: ^{1}H NMR (400 MHz, D_2O, CD_3COOD) δ ppm: 2.08 (H-Ac, s), 3.23 (H-2, s), 3.48–4.08 (H-3-H-6, m, 5H), 4.89 (H-1, partially overlapped with the solvent peak, s, 1H), 6.74 (H-7, d), 6.90 (H-11, t), 7.51 (H-10, H-12, t), 7.69 (H-8, d), 8.02 (H-9, H-13, d).

p-Coumaric acid conjugated chitosan: ^{1}H NMR (400 MHz, D_2O, CD_3COOD) δ ppm: 2.08 (H-Ac, s), 3.21 (H-2, s), 3.48–4.08 (H-3-H-6, m, 5H), 5.06 (H-1, partially overlapped with the solvent peak, s, 1H), 6.58 (H-7, d), 6.94 (H-10, H-11, d), 7.53 (H-9, H-12, d), 7.59 (H-8, d).

Caffeic acid conjugated chitosan: ^{1}H NMR (400 MHz, D_2O, CD_3COOD) δ ppm: 2.08 (H-Ac, s), 3.05 (H-2, s), 3.17–4.02 (H-3-H-6, m, 5H), 4.91 (H-1, partially overlapped with the solvent peak, s, 1H), 6.20 (H-7, d), 6.78 (H-9, d), 6.95 (H-10, d), 7.02 (H11, s) 7.46 (H-8, d).

Ferulic acid conjugated chitosan: ^{1}H NMR (400 MHz, D_2O, CD_3COOD) δ ppm: 2.08 (H-Ac, s), 3.20 (H-2, s), 3.32–4.24 (H-3-H-6, m, 5H), 3.77 (O-CH_3, overlapping with chitosan peaks) 4.94 (H-1, partially overlapped with the solvent peak, s, 1H), 6.71 (H- 7, d), 7.48 (H-9 and H-10 merge), 6.95 (H-10, d), 7.60 (H11, s) 7.66 (H-8, d).

2.6 Characterization of antioxidant-chitosan conjugates

2.6.1 ^{1}H NMR spectroscopy

^{1}H NMR samples were measured with Bruker AVANCE 400 (Bruker Biospin GmbH, Karlsruhe,

Germany) operating at 400 MHz at 298 K. In the case of the chitosan containing samples, the *N*-acetyl peak (2.08 ppm) was used as the internal reference with D_2O, D_2O/CD_3COOD, and D_2O/DCl solvents. In the case of the HCA samples, D_6-DMSO and $CDCl_3$ solvents were utilized, and the chemical shifts were calculated with reference to the residual hydrogen signal of the corresponding deuterated solvent. The concentration of the samples ranged between 0.5 and 15 mg/mL. *Topspin* software (Bruker, Germany) was utilized to interpret the spectra. The integral values were used to estimate the DS of the precursors and the derivatives. The degree of acetylation (DA) for chitosan was calculated from the ratio of the integral for the six H-2, H-3, H-4, H-5, H-6, and H-6' protons on the sugar backbone relative to the acetyl peak (HAc).

The DS of the conjugates was determined from the ratio of the integral for the six H-2, H-3, H-4, H-5, H-6 and H-6' protons relative to the integral of the aromatic and alkenyl protons of the antioxidant moiety.

Equation 1: DS for acetylation of chitosan (Degree of acetylation, DA):

$$DS = \left[\frac{\int HAc}{\int H2 - H6} x \frac{6}{3}\right] x100$$

DS= Degree of substitution

HAc= C=O(CH_3) protons of chitosan

H2-H6= H-2, H-3, H-4, H-5, H-6, H-6' protons of chitosan

Equation 2: DS for TBDMS protection of chitosan:

$$DS = \left[\frac{\int 6(CH3)}{\int H1 - H6} x \frac{7}{18}\right] x100$$

6(CH_3)= $(CH_3)_2C$ protons

H1-H6=H-1, H-2, H-3, H-4, H-5, H-6, H-6' protons of chitosan

Equation 3: DS for cinnamic acid conjugation to chitosan:

$$DS = \left[\frac{\int H7 - H13}{\int H2 - H6} x \frac{6}{7}\right] x100$$

H7-H13= H7, H8, H9, H10, H11, H12, H13 cinnamic acid protons

H2-H6= H-2, H-3, H-4, H-5, H-6, H-6' protons of chitosan

Equation 4: DS for *p*-coumaric acid conjugation to chitosan:

$$DS = \left[\frac{\int H7 - H12}{\int H2 - H6} x \frac{6}{6}\right] x100$$

H7-H12= H7, H8, H9, H10, H11, H12 *p*-coumaric acid protons

H2-H6= H-2, H-3, H-4, H-5, H-6, H-6' protons of chitosan

Equation 5: DS for ferulic acid conjugation to chitosan:

$$DS = \frac{6}{\int II\left(\frac{5}{\int I} - \frac{3}{\int II}\right)} = \frac{6\int I}{5\int II - 3\int I}$$

∫I= ∫H9-H11 ferulic protons

∫II =∫H2-6,OCH_3 - H-2, H-3, H-4, H-5, H-6, H-6' protons of chitosan and OCH_3 ferulic protons

(details in the supplementing information)

Equation 6: DS for caffeic acid conjugation to chitosan:

$$DS = \left[\frac{\int H7 - H11}{\int H2 - H6} x \frac{6}{5}\right] x100$$

H7-H11= H7, H8, H9, H10, H11 caffeic acid protons

H2-H6= H-2, H-3, H-4, H-5, H-6, H-6' protons of chitosan

2.6.2 DPPH scavenging assay

The DPPH radical scavenging activity was evaluated using the method previously reported by Moreno-Vasquez et al., with slight modifications (Moreno-Vasquez et al., 2017). A 1:1 mix of 1% v/v acetic acid solution in water and MeOH solvent system was used to dissolve the samples by stirring overnight (chitosan: 11232 µg/mL, HCA-s and conjugates: 5616 µg/mL). The samples were then diluted in MeOH to give dilution series in reagent tubes with a two-fold dilution interval between samples. 2500 µL DPPH solution (0.1mM in MeOH) was added to 500 µL of each sample, vortexed, and allowed to stand in darkness at room temperature for 1 h. L-ascorbic acid was used as positive control. The absorbance was measured at 517 nm using an Ultrospec 2000 pro UV/Visible spectrophotometer. The solvent was used as the blank without DPPH.

Solvent+DPPH was used as the control. The scavenging activities were expressed as the percent inhibition of DPPH and calculated with the following equation:

$$\text{Inhibition \%} = \left(1 - \frac{Abs1 - Abs2}{Abs0}\right) \times 100$$

where Abs_1 is the absorbance of the corresponding sample, Abs_2 is the absorbance of the sample without DPPH solution, and Abs_0 is the absorbance of the control. The absorbance values were plotted against the logarithmic concentration in a semi-logarithmic graph to determine the half-maximal effective concentration (EC_{50}) values. Sigmoidal curve-fitting was performed in Kaleidagraph with the equation $Y=m_1+(m_2-m_1)/(1+(X/m_3)^{m_4})$, where m_1 is the Y_{min} value, m_2 is the Y_{max} value, m_3 is the X value at mid-point of Y (EC_{50}), m_4 is the slope of the curve at the midpoint.

2.6.3 Antibacterial activity

The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) were measured according to the Clinical and Laboratory Standards Institute standard procedure (CLSI, 2006). The media used for the MIC test was Mueller Hinton broth (adjusted to pH 5.5 with HCl) and Mueller Hinton agar for the MLC. The samples were dissolved in 5% v/v DMSO in water at a concentration of 8192 µg/mL. 50 µL of broth was added to wells 2-12 of a micro-titer tray, then 50 µL of sample solution was added to the first two wells, and a double dilution series was prepared from well 2-10. Bacterial solutions of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213) were prepared at 0.5 MacFarland (approximately $1\text{-}2*10^8$ bacteria/mL), then diluted 100 times and 50 µL of the diluted solution added to wells 1-11 ($\sim 5*10^5$ bacteria/mL). A viable cell count was used to confirm the number of bacteria in the tests. Gentamicin was used as performance control, broth without conjugates as growth control (well 11), and broth without bacteria and conjugates served as a sterility control (well 12). The microtiter trays were incubated at 36 °C for 24 h. The MLC measurement was carried out after determining the MIC value. After establishing the MIC value, 10 µL from each dilution where inhibition is observed was subcultured to a Mueller Hinton agar medium. The plates were incubated overnight at 36 °C, the colony-forming units (CFU) were counted, and MLC determined.

2.7 Design of experiment (DoE)

The effect of three factors (cinnamoyl chloride eq, TEA eq, time) were investigated on the DS

response with the help of MODDE software (Sartorius GmbH). A full factorial design (2^3) was carried out in Multiple Linear Regression (MLR) mode, using the equation:

$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\varepsilon$

Y is the response (DS), the X_1, X_2 and X_3 are the three experimental factors, the β-s represent regression coefficients that will be estimated from the experimental data and ε is the random variance (Thorsteinsdóttir & Thorsteinsdóttir, 2021). An orthogonal (balanced) design was carried out with all combinations of the factor levels (8 runs). Additionally, three replicates were performed to investigate the reproducibility.

3. Results and discussion

3.1 Synthesis of chitosan derivatives

The antioxidants investigated in this study were cinnamic acid and cinnamic acid derivatives. These can be linked to chitosan through amide bonds that can be formed by condensing the carboxylic acid and the primary amino groups of chitosan. The carboxylic acid can be activated for this reaction by forming acyl halides, activated esters, or anhydrides (Valeur & Bradley, 2009). Six published methods were tested (Table 1) as possible procedures for the current study. The procedures were based on carbodiimide activation, sometimes also forming activated ester intermediates.

Table 1: Six experiments were carried out based on literature methods

Method based on:	Cinnamic acid (eq)	Reagent (eq)	Time (h)	Temp. (°C)	Solvent	DS (%)*
Woranuch et al. (2013)	1	EDC (1)	3	60	EtOH	< 0.1 %
Wang et al. (2015)	1	EDC (1.2)/NHS (1.2)	24	25	DMSO/H_2O	6.4
Schreiber et al. (2013)	20	EDC (1)/NHS (1)	24	25	EtOH/H_2O	7.6
Eom et al. (2012)	1	DCC (1)/HOBt (1)/TEA (3)	24	25	MeOH/H_2O	< 0.1 %
Pasanphan et al. (2008)	1	EDC (1)/NHS (1)	24	25	EtOH	0.6
Xie et al. (2014)	1	EDC (1)/HOBt (1)	24	25	H_2O	0.4

*These DS values were determined in our laboratory (using ^{1}H-NMR spectroscopy) after performing the syntheses from the literature. Xie et al., Schreiber et al. (gallic acid conjugates) and Wang et al. (cinnamic acid conjugate) did not report the DS. Pasanphan et al. made gallic acid conjugates and reported DS 15% from elemental analysis. Eom et al. prepared eight different conjugates and reported the DS (5-10%) from the total phenolic content using Folin - Ciocalteu reagent. Woranuch et al. used ^{1}H-NMR and reported 37% DS for ferulic acid conjugates.

In our experiments following literature procedures (Wang & Kim, 2015; Xie, Hu, Wang, & Zeng, 2014), using cinnamic acid and the available chitosan material, we only obtained conjugates with

low DS (< 8%), even when a large excess of cinnamic acid (20 eq) was used (Schreiber, Bozell, Hayes, & Zivanovic, 2013). Some of the studies did not report the DS or used IR or elemental analysis, which are not considered as accurate as ^{1}H-NMR for this purpose and are more affected by contamination (Másson, 2021b).

Woranuch et al. reported 37% DS calculated from ^{1}H-NMR for ferulic acid conjugates. However, they did observe a shift in the vinylic and aromatic proton peaks relative to free ferulic acid in their conjugate. When we used this method for cinnamic acid conjugation, the DS for the conjugate was <0.1% according to NMR. Furthermore, these methods may cause partial polymerization of HCA-s through ester formation (Kaneko, Kinugawa, Matsumoto, & Kaneko, 2010). Thus, it was not considered feasible to use these literature methods as a general procedure to synthesize conjugates for the current study.

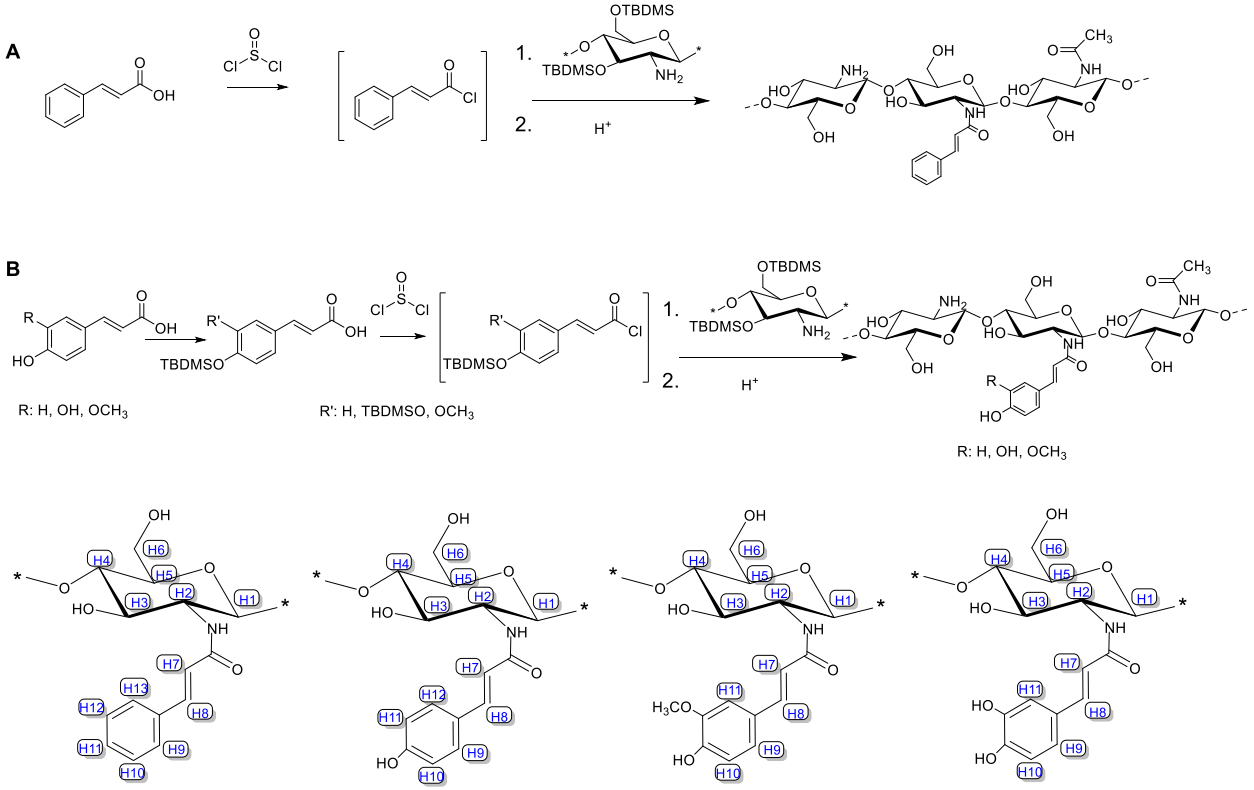

Figure 1: Synthesis scheme for the HCA-chitosan conjugates and the numbering of the protons that could be observed in the ^{1}H-NMR spectra.

The conjugation was, therefore, done using TBDMS-chitosan. This required additional reaction steps for protecting the starting materials but offered the advantage of fully selective reactions carried out in organic solvents, avoiding competing hydrolysis of the activated intermediates.

Previous work has reported the reaction of HCA-s with amines with TEA as the base and DCM as solvent (Okamoto et al., 2009). The TBDMS-protection of HCA-s was carried out by a published method (Matsuno et al., 2001).

Cinnamic acid was a good candidate to develop the conjugation procedure as it does not possess aromatic hydroxyl groups that need to be protected. Cinnamic acid was converted to cinnamoyl chloride and reacted with the 3,6-di-*O*-TBDMS-chitosan using TEA as the base (Figure 1A). The cinnamic acid conjugated TBDMS-chitosan was then deprotected by stirring in 2M HCl solution in MeOH, followed by ion exchange, dialysis and lyophilization to obtain the cinnamic acid conjugate. A full factorial design optimization (Anderson & McLean, 2018) with three factors (cinnamoyl chloride equivalents, TEA equivalents, and time) on two levels was carried out (Table 2). The highest DS with the newly developed method, using only one eq of cinnamoyl chloride, was 15%. This was a two-fold increase in the DS compared to the tested published method, where 20 eq of cinnamic acid was used with carbodiimide coupling (Table 1). The resulting DS of the conjugates correlated with the equivalent ratio but was not influenced by the quantity of the TEA or reaction time. Therefore, the eq of cinnamoyl chloride was the only significant factor. With the increase of the acyl chloride eq, higher DS could be obtained, and the eq of base and reaction time could be decreased without negatively affecting the outcome of the reaction. Three replicates were carried out (1B, 3B, 7B) with a commercial cinnamoyl chloride. It was found that the model had good reproducibility and that synthesized cinnamoyl chloride was equally useful as a reagent as the commercially available cinnamoyl chloride (whereas the acyl chlorides of protected HCA are not available).

Table 2: Design of Experiment - Full Factorial Design

Exp.	Cinnamoyl chloride (eq)	TEA (eq)	Time (h)	DS (%)
1	0.25	2	24	9
2	1	2	24	15
3	0.25	7.6	24	9
4	1	7.6	24	13
5	0.25	2	48	9
6	1	2	48	15
7	0.25	7.6	48	8
8	1	7.6	48	15
Using commercial cinnamoyl chloride reagent				
1B	0.25	2	24	10
3B	0.25	7.6	24	10

7B	0.25	7.6	48	7
Using excess cinnamoyl chloride				
9	2	2	24	36
10	3	2	24	60

The highest obtained DS was 60%, using three eq cinnamoyl chloride, which showed that the DS could be increased by using excess cinnamoyl chloride.

The optimized method was applied to synthesize the remaining conjugates with the TBDMS-protected HCA-s as well (Figure 1B). The corresponding acyl chlorides were applied in 1, 2, and 3 equivalents, providing conjugates with different DS values. Table 3 displays the results of the syntheses. The aim to synthesize conjugates with different DS values was fulfilled. Much higher DS values were obtained with the newly developed method compared to the tested literature methods (Table 1). However, in order to get high DS, an excess of acyl chloride was needed. Thus, the conversion was less than 100%. This could be due to the unstable nature of acyl chlorides. The efficiency was similar to cinnamoyl chloride for the TBDMS-coumaroyl chloride but about half with TBDMS-feruloyl chloride and di-TBDMS-caffeoyl chloride.

Table 3: HCA-chitosan conjugate syntheses

Exp.	Reagent	Acyl chloride (eq)	DS (%)	Weight ratio (%)
11	TBDMS-Coumaroyl chloride	1	9	13
12	TBDMS-Coumaroyl chloride	2	35	51
13	TBDMS-Coumaroyl chloride	3	40	59
14	TBDMS-Feruloyl chloride	1	3	5
15	TBDMS-Feruloyl chloride	2	17	30
16	TBDMS-Feruloyl chloride	3	27	48
17	di-TBDMS-Caffeoyl chloride	1	5	8
18	di-TBDMS-Caffeoyl chloride	2	13	21
19	di-TBDMS-Caffeoyl chloride	3	21	34

3.2 Characterization

3.2.1 ^{1}H NMR spectroscopy

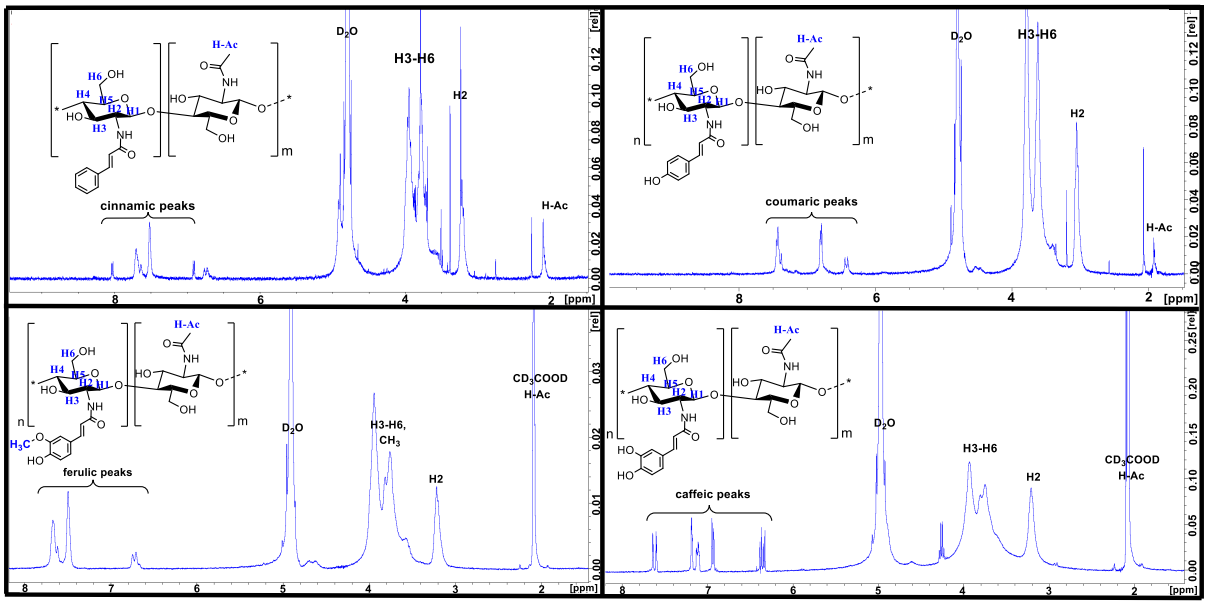

Figure 2: ^{1}H NMR spectra of cinnamic acid conjugated chitosan (DS=10%), p-coumaric acid conjugated chitosan (DS=9%), ferulic acid conjugated chitosan (DS=17%) and caffeic acid conjugated chitosan (DS=13%)

To confirm the new compounds were true conjugates, the ^{1}H-NMR spectra of the conjugates were collected (Figure 2), then a sample of mixed native chitosan and HCA-s were submitted for analysis as well. These were compared to see if there was a chemical shift, which would confirm the synthesis of true conjugates as the characteristic antioxidant peaks were shifted 0.01-0.66 ppm relative to the acids. (Table 4).

Table 4: The change in chemical shift of H-7, H-8, and H-9 protons in chitosan conjugated HCA relative to HCA

Compound	ΔH-7 (ppm)	ΔH-8 in (ppm)	ΔH-9 (ppm)
Cinnamic acid chitosan conjugate	0.21	-0.08	0.11
p-Coumaric acid chitosan conjugate	0.08	-0.1	-0.1
Ferulic acid chitosan conjugate	0.3	0.03	0.57
Caffeic acid chitosan conjugate	-0.04	-0.01	0.66

3.3 Antioxidant activity

Finding a suitable solvent for the DPPH assay (Figure 4) has proven challenging. While chitosan and the conjugates were soluble in aqueous solutions (1% v/v acetic acid), the HCA-s were found insoluble. The addition of MeOH to the 1% v/v acetic acid solution has fully dissolved the starting materials and the conjugates, except for one *p*-coumaric acid conjugate (12) sample. Cinnamic acid did not exhibit DPPH scavenging activity in the tested concentrations (Table 5). This was expected as aromatic core lacks an OH group that can participate in a hydrogen transfer reaction with the free radical (Szeląg, Urbaniak, & Bluyssen, 2015; Teixeira, Gaspar, Garrido, Garrido, & Borges, 2013). The cinnamic acid conjugates also lacked activity. The EC_{50} of unmodified chitosan was found to be 2777 μg/mL. *p*-Coumaric acid, which has a p-OH group on the phenyl moiety, and *p*-coumaric acid chitosan conjugates exhibited DPPH scavenging activity, but this was less than 50% in the tested concentrations, so the EC_{50} could only be estimated by fitting to a sigmoidal curve. Ferulic acid, which has a p-OH and a m-metoxy group that further enhances the activity, exhibited significant antioxidant activity (EC_{50}=5 μg/mL), with nearly full reduction of DPPH at ≥39 μg/mL concentration. The ferulic acid-chitosan conjugates also exhibited high DPPH scavenging activities (EC_{50} values between 107-982 μg/mL), which could be correlated with the DS of the conjugate. When multiplied with the weight ratio (WR or grafting ratio) for the antioxidant moiety, it was found that the EC_{50} was about 10 times less than for the free ferulic acid. Thus, it could be concluded that conjugation to chitosan reduces the antioxidant activity in this case, indicating that conversion from acid to amide changes the electron resonance structure in the whole system.

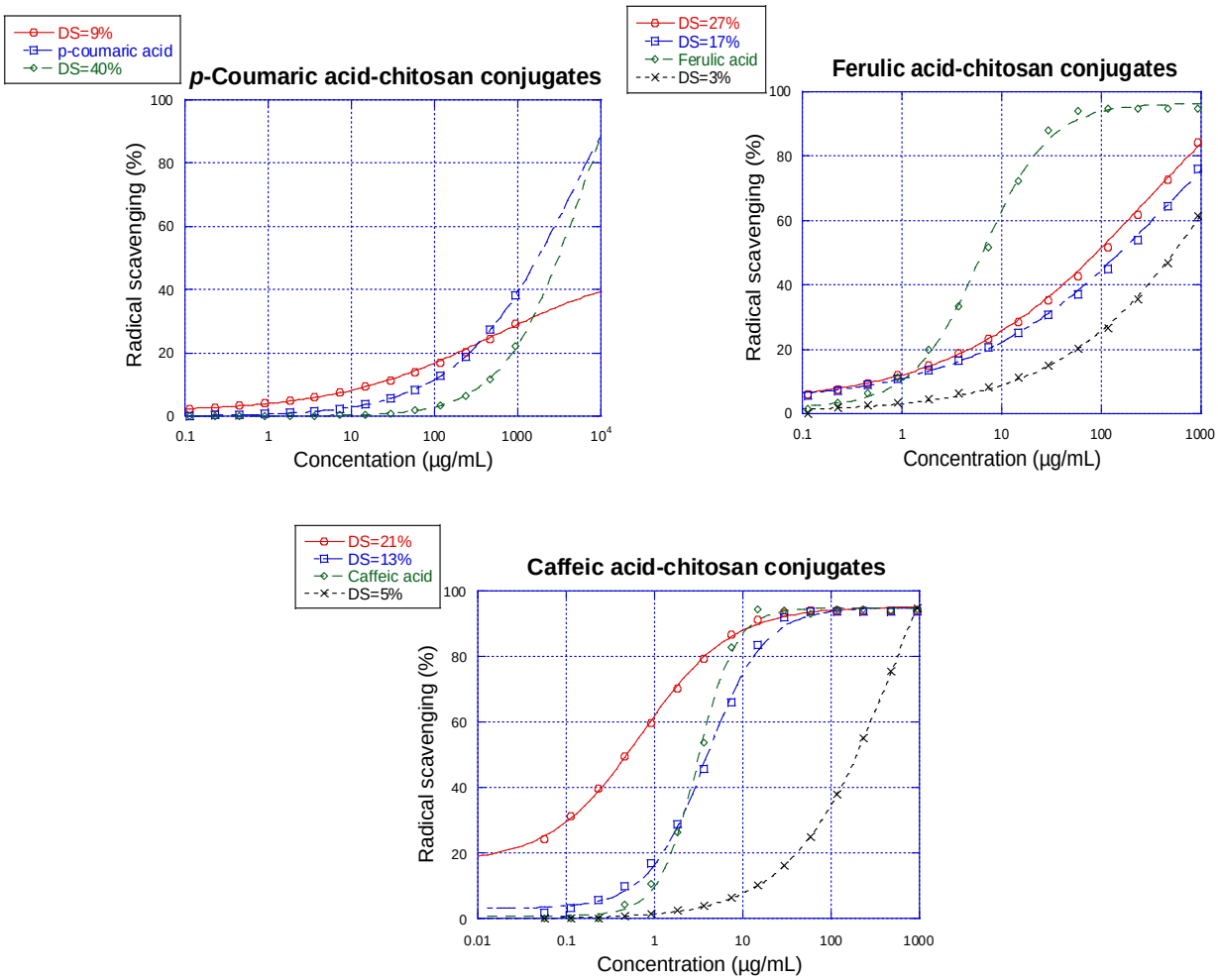

Figure 4: DPPH scavenging activity of HCA-s and their respective chitosan conjugates

Caffeic acid (EC_{50}=2 µg/mL), which has p and m OH groups, and the caffeic acid-chitosan conjugates exhibited high DPPH scavenging activities (EC_{50} values between 0.7-406 µg/mL), and this correlated strongly with the DS. Based on the WR, the activity increased about 160-fold from the DS 5% conjugate to the DS 21% conjugate. Hence the conjugation of caffeic acid to chitosan could increase the antioxidant activity relative to the starting materials and give a potent antioxidant compound with significant DPPH scavenging activity (EC_{50}=0.7 µg/mL). This was an unexpected result. It is thus possible, that intramolecular hydrogen bonding between di-hydroxylated aromatic moieties contributes to increased antioxidant activity. Our findings that unmodified chitosan does not possess significant antioxidant activity and conjugation of HCA confers a high activity to the polymer is consistent with previous reports (Pasanphan & Chirachanchai, 2008).

398

Table 5: Antioxidant activity

Name	DS (%)	EC_{50} (µg/mL)	EC_{50}xWR/100 (µg/mL)
Chitosan		2777±0.1	
Ascorbic acid		7±0.8	
Cinnamic acid		≥10000*	
p-Coumaric acid		1503±107*	
Ferulic acid		5±0.3	
Caffeic acid		2±0.08	
Cinnamic acid-chitosan conjugate (2)	15	≥10000*	
Cinnamic acid-chitosan conjugate (9)	36	≥10000*	
Cinnamic acid-chitosan conjugate (10)	60	≥10000*	
p-Coumaric acid-chitosan conjugate (11)	9	2989±112*	389
p-Coumaric acid-chitosan conjugate (13)	40	1735±62*	885
Ferulic acid-chitosan conjugate (14)	3	982±59	49
Ferulic acid-chitosan conjugate (15)	17	470±17	141
Ferulic acid-chitosan conjugate (16)	27	107±8.1	51
Caffeic acid-chitosan conjugate (17)	5	406±24	32
Caffeic acid-chitosan conjugate (18)	13	4±0.2	0.8
Caffeic acid-chitosan conjugate (19)	21	0.70±0.05	0.2

*DPPH scavenging at 5616 µg/mL: cinnamic acid=6%; *p*-coumaric acid: 38%; conjugates 2,9 and 10=0%; conjugate 11=22%; conjugate 13=30%

399

400 **3.4 Antibacterial activity**

401 The antibacterial activity of the chitosan starting material and the synthesized conjugates
402 were investigated against gram-negative *E. Coli* and gram-positive *S. aureus*. 5% v/v DMSO
403 in water was chosen as a solvent since the HCA-s did not dissolve in 1% acetic acid solution
404 (8192 µg/mL). The MIC and MLC of the compounds were measured at pH 5.5, and the
405 results are displayed in Table 6. The MLC values were equal to the MIC values in all cases,
406 suggesting that all conjugates and starting materials are bactericidal. All controls showed
407 correct results.

408

Table 6: Antibacterial activity against *S. aureus* and *E. coli*

Compound	DS (%)	MIC/MLC values (µg/mL)*	
		S.aureus	*E.coli*
Chitosan		256	256
Cinnamic acid		512	1024

p-Coumaric acid		512	512
Ferulic acid		512	1024
Caffeic acid		1024	1024
Cinnamic conjugate (7B)	7	256	128
Cinnamic conjugate (1)	9	128	64
Cinnamic conjugate (2)	15	128	128
Cinnamic conjugate (9)	36	≥2048	512
Cinnamic conjugate (10)	60	≥2048	≥2048
p-Coumaric conjugate (11)	9	256	128
p-Coumaric conjugate (12)	35	1024	512
p-Coumaric conjugate (13)	40	1024	256
Ferulic conjugate (14)	3	256	256
Ferulic conjugate (15)	17	1024	256
Ferulic conjugate (16)	27	1024	1024
Caffeic conjugate (17)	5	1024	64
Caffeic conjugate (18)	13	512	1024
Caffeic conjugate (19)	21	≥2048	≥2048

*The measured MIC and MLC values were the same in every case.

The antibacterial effect was confirmed for the chitosan starting material with the MIC and MLC value 256 µg/mL against both pathogens. The HCA-s were also active with MIC and MLC between 512 and 1024 µg/ml. Some of the chitosan conjugates with DS <20% appeared to be more active than chitosan with MIC/MLC equal to 128 µg/ml. However, this is only a one dilution difference and can therefore not be considered significant. Only in two cases, with DS 9% cinnamic acid conjugate and DS 5% caffeic acid conjugate against *E. coli*, the value was 64 µg/ml, a two-dilution difference that can be considered significant. In contrast, there was a clear trend toward decreased activity when the DS was >20%. The highest DS cinnamic acid and caffeic acid conjugates were inactive in the tested concentrations. Thus, the conjugation of HCA may decrease antibacterial activity, especially when the DS is above 20%.

These results showed that HCA substituents do not contribute to antibacterial activity. They will reduce the number of quaternized primary amino groups and this will reduce their activity similar to the effect of an increase in the degree of acetylation (DA) on the antibacterial activity of chitosan (Younes, Sellimi, Rinaudo, Jellouli, & Nasri, 2014) and chitosan derivatives (Rathinam, Ólafsdóttir, et al., 2020; Sahariah, Snorradóttir, Hjálmarsdóttir, Sigurjónsson, & Másson, 2016). Highly lipophilic substituents, similarly to cinnamic acid, can reduce the activity (Rúnarsson et al., 2010), possibly by causing self-association on the polymer chains. Our investigation has also shown that hydroxypropyl substituents, which are hydrophilic and can

form hydrogen bonds similar to the caffeic moiety, will also have a strong negative effect on the antibacterial activity (Másson, 2021a; Rathinam, Solodova, Kristjánsdóttir, Hjálmarsdóttir, & Másson, 2020b).

4. Conclusions

A new synthetic method was developed for the synthesis of HCA-chitosan conjugates based on TBDMS-protection and reaction with acyl chlorides to form amide linkages. The method was optimized with DoE and 19 conjugates with DS ranging from 5% to 60% were synthesized. The cinnamic acid conjugated chitosans had no DPPH scavenging activity in the tested concentrations and unmodified chitosan had weak antioxidant activity (EC_{50}=2777 μg/mL). In contrast, the HCA-chitosans possessed enhanced activities that correlated with the DS. The caffeic acid chitosan conjugates possessed the strongest scavenging activity (EC_{50} 0.7, 4, 406 μg/mL corresponding to DS 21, 13, 5%, respectively), followed by the ferulic- (EC_{50} 107, 470, 982 μg/mL with DS 27, 17, 3%, respectively) and *p*-coumaric acid conjugates (EC_{50} 1503, 2989 μg/mL with DS 40, 9%, respectively). Furthermore, the caffeic acid chitosan conjugate with DS=21% exhibited stronger DPPH scavenging activity than caffeic acid itself. The antibacterial activity against gram-negative *E. Coli* and gram-positive *S. aureus* were tested. The MIC value was equal to MLC in all cases. Chitosan possessed good antibacterial activity against both strains, namely 256 μg/mL MIC. Conjugates with low DS had activity comparable to chitosan. The antibacterial activity was reduced when the DS was higher than 20%. Future perspectives for the work include further DoE designs to maximize the efficacy of each step and have more control over the DS.

Supplementary data

There is supporting information for this article.

Authors Information

Már Másson (MM) designed the research plan and supervised the project. The synthesis work and characterization were done by Vivien Nagy (VN) with assistance from Priyanka Sahariah (PS). The antimicrobial assay was done by VN, supervised by Martha Á. Hjálmarsdóttir (MH).

VN did the antioxidant assay. VN and MM prepared the manuscript, and the final version was participated in interpreting the results and approved by all co-authors. This work presents no conflict of interest for any of the authors.

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