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Research Article

SYNTHESIS, CHARACTERIZATION, ANTIOXIDANT AND ANTIBACTERIAL PROPRIETIES OF CHITOSAN ASCORBATE

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ABSTRACT

In this study, chitosan ascorbate (CHAA) was synthesized through the Schiff base reaction and characterized with Fourier transformed infrared spectroscopy (FTIR), the solubility of CHAA in distilled water was greatly improved. The antioxidant activity of CHAA compared to chitosan (CH) was performed by four different analytical assays (DPPH, TBARS, NO, H_2O_2); the results showed that CHAA exhibited a higher antioxidant activity than that CH. The minimum inhibitory concentrations of CH and CHAA against five bacteria (*E. coli*, *P. aeruginosa*, *S. Typhimurium*, *L. monocytogenes*, *S. aureus*) were tested; the antibacterial efficacy of CHAA is lower than that of CH because of the consumption of some amine groups on chitosan during the grafting process. However, CHAA still possesses antibacterial efficacy against both bacteria. Combining chitosan and ascorbic acid improve the antioxidant activity of CH, and still possesses antibacterial efficacy against both bacteria.

Keywords: Chitosan, Ascorbic Acid, FTIR, Antioxidant, Antibacterial.

1. INTRODUCTION

Chitosan (CH), the linear polymer of D-glucosamine in b (1–4) linkage, has been recommended as a cell-protective molecule due to its biocompatibility, biodegradability, non-toxicity, adsorption properties, and regulation of cell activation[1, 2]. Recently, the antioxidant property of chitosan has attracted increasing attention[3, 4]. This oligosaccharide possesses additional characteristics such as immune enhancing, anti-inflammatory effects [4], and enhancing protection against infection with some pathogens in mice[5, 6], anti fungal[7, 8], and antimicrobial activities[9]. But the application of CH in biomedical fields is limited owing to the poor solubility in physiological media. Through chemical modification, CH derivatives of specific functions (i.e. improved solubility and bioactivity) can be obtained by introducing active groups on to the hydroxyl (C3 and C6) and amino groups (C2) of CH molecules. Studies suggest that amino groups on the surface of CH chains are reactive enough to react with a number of acid chlorides, acid anhydrides and aldehydes[10].

Ascorbic acid (AA), as a popular antioxidant, The ability of AA to scavenge free radicals, promote collagen biosynthesis, cause melanin reduction, provide photo-protection and enhance immunity, makes it widely applicable in the food, cosmetic and pharmaceutical industries[11, 12]. However, AA is not just an antioxidant. Its remarkable function as a co-substrate of many important dioxygenases, in regenerating enzymes and its intriguing function in gene expression have been reviewed by Arrigoni[13]. But it is highly unstable and very easy to get oxidized and changes to dehydroascorbic acid when exposed to light, air and elevated temperature. To increase the stability of AA, various derivatives have been synthesized, including the metal salts (Na, Ca salts), ethers, esters and the polysaccharide derivatives[14].

In this study we aimed to investigate the antimicrobial and antioxidant activities of chitosan ascorbate (CHAA). For this, CHAA was first synthesized via the Schiff base reaction. The

synthesis product was characterized by Fourier Transform Infrared Spectroscopy (FTIR), and the solubility, antioxidant and antimicrobial activities between CH and CHAA were compared.

2. MATERIALS AND METHODS

Chitosan (DDA > 75%) and all chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (St. LouisMo).

2.1. Preparation of chitosan ascorbate

Chitosan ascorbate (CHAA) was prepared as reported by Muzzarelli et al[15]. Briefly, 1% (w/v) chitosan (CH) powder was suspended in distillated water, then 100% (w/w) of ascorbic acid (AA) was added (under nitrogen to remove dissolved oxygen from solutions), resulting in the immediate dissolution of chitosan. After 6 hours of stirring, the solution was precipitated with acetone, and the raw product was dialyzed against water for 3 days. The chitosan ascorbate (CHAA) was obtained after freeze-drying. The possible reaction mechanism is shown in figure1.

2.2. Fourier transform infrared spectroscopy (FTIR)

The chemical composition and the difference between CH and CHAA were obtained using the PerkinElmer Spectrum Two ATR-FTIR, over the wave number range between 4000 and 400 cm^{-1}.

2.3. Apparent solubility testing

According to the general notices of United States pharmacopeia, 100 mg of CHAA as added into 10 ml of water and different solvents respectively and stirred vigorously at 25°C for 24 h to observe its apparent solubility based on the transparency of solutions.

2.4. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The free radical scavenging effect of CH and CHAA was estimated according to the method of Shimada et al[16]. Briefly 1 mL of each sample (0.2, 0.4, 0.6, 0.8, 1 mg/mL) was mixed with 3 mL of methanolic solution of DPPH (300 µM). The reaction mixture was vortexed and incubated for 30 min in room temperature. The absorbance of the solution was measured at 517 nm. Ascorbic acid was used as standard. The inhibitory percentage of DPPH was calculated using to the following equation:

$$DPPH\ Scavenging\ effect\ (\%) = [1- (Abs_{sample}/Abs_{control})] \times 100 \ldots\ldots\ldots\ldots\ldots (1)$$

Where Abs_{sample} represents the absorbance of the chitin and chitosan solution and $Abs_{control}$ represents the absorbance of DPPH solution without the addition of the samples. All experiments were carried out in triplicate.

2.5. Linoleic acid peroxidation with TBARS assay

In this assay antioxidant capacity is determined by measuring the TBARS arising from linoleic acid peroxidation[17]. The reaction mixture contained 500 µl linoleic acid (20 mM), 500 µl Tris HCl (100 mM, pH 7.5), 100 µl $FeSO_4{}^*7H_2O$ (4 mM) and a varying concentration of each sample (0.2, 0.4, 0.6, 0.8, 1 mg/mL). Linoleic acid peroxidation was initiated by the addition of 100 µl of ascorbic acid (2 mM, Fenton reaction), incubated for 30 min at 37 °C and achieved by the addition of 2 mL trichloroacetic acid (10%). Therefore 1 mL of the mixture was added with 1 mL of thiobarbituric acid (1% in 50 mM NaOH), followed by heating for 10 min at 95°C. The mixtures were centrifuged at 3500/g for 10 min and the absorbance of thiobarbituric acid-reacting substances (TBARS) in the supernatant was measured at 532 nm. Gallic acid was used as standard. The percentage of antioxidant activity is determined using the following equation:

$$Linoleic\ acid\ peroxidation\ inhibition\ (\%) = [(Ac-As) / (Ac-An)] \times 100 \ldots\ldots\ldots (2)$$

Ac = Absorbance of control (without extract)
As = Absorbance of extract
An = Absorbance of blank (without extract and $FeSO_4{}^*7H_2O$).
All experiments were carried out in triplicate.

2.6. Nitric oxide radical scavenging assay

The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. 2 mL of different solution was mixed with sodium nitroprusside (4 mL of 10 mM, in PBS pH 7.4), and incubated at room temperature for 150 min. After the incubation period, 0.5 mL of Griess reagent was added[18]. The absorbance of the chromophore formed was measured at 546 nm. All the reactions were performed in triplicate and the Nitric oxide scavenging activity was calculated using the following equation:

$$Nitric\ oxide\ scavenging\ activity\ (\%) = [1- (Abs_{sample} / Abs_{control})] \times 100 \ldots\ldots\ldots (3)$$

Where ($Abs_{control}$) is the absorbance of the control and (Abs_{sample}) is the absorbance of the sample solution.

2.7. Hydrogen peroxide radical scavenging assay

Hydrogen peroxide radical scavenging activity of edible films was determined using the method reported by Ruch et al[19]. A solution of H_2O_2 was prepared in phosphate buffer (pH 7.4). 3 mL of (40 mM H_2O_2) solution was mixed with 500 µL of different methanolic solution. After incubation at 37°C for 10 minutes absorbance was measured at 230 nm. Blank solutions were taken using phosphate buffer without H_2O_2. For each concentration a separate blank sample was used for background subtraction. All the tests were performed in triplicate. The H_2O_2 scavenging activity was calculated using the following equation:

$$H_2O_2 \text{ scavenging activity (\%)} = [1 - (Abs_{sample} / Abs_{control})] \times 100 \quad (4)$$

Where ($Abs_{control}$) is the absorbance of the control and (Abs_{sample}) is the absorbance of the sample solution.

2.8. Antimicrobial Activity

Antibacterial activities of CH and CHAA were examined as the inhibitory effects against the growth of gram-positive and gram-negative bacteria: (*Escherichia coli ATCC, Pseudomonas aeruginosa ATCC, Salmonella Typhimurium ATCC, Listeria monocytogenes ATCC, Staphylococcus aureus ATCC*). All bacteria were obtained from culture collection of the laboratory of microbiology, Faculty of pharmacy (Monastir, Tunisia).

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) are important to confirmed resistance of bacteria and fungi to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. MIC of CH and CHAA was determined using the method reported by Ellof[20]. The inocula of the bacterial strains were prepared from 12 h broth cultures and suspensions were adjusted to 5 × 10^5 CFU/mL. CH was dispersed in a 1% acetic acid solution and CHAA was dispersed in distillated water, at a concentration of 10 mg/mL before being applied to broth and each solution was diluted (serial two-fold dilutions) to give final CH and CHAA concentrations 5000, 2500, 1250, 625, 312.5, 156.25 and 78.125, 39.08, 19.54 ppm, respectively.

In brief, the 96-well plates were prepared by dispensing into each well 95 µl of nutrient broth (Mueller-Hinton), and 50 µl of the inocula, and 100 µl of varying concentration of each sample. The last well without compound were used as negative control. The final volume in each well was 200 µl. The plate was covered with a sterile plate sealer and then incubated for 24 h at 37°C. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of micro-organisms, after incubation[21].

3. RESULTS AND DISCUSSIONS

3.1. Solubility test

CH is soluble in dilute aqueous acidic solutions below its pKa (6.3), in which it can convert glucosamine units ($-NH_2$) into the soluble protonated form ($-NH^{+3}$)[22]. But CH is insoluble in water and most common organic solvents. The insolubility is a result of its extensive intramolecular and intermolecular H-bonding between the chains and sheets of CH[23]. Solubility tests showed that CHAA was also insoluble in common organic solvents, such as (DMSO, methanol, ethanol, acetonitrile and chloroform). But the solubility of CHAA in distilled water was better improved because most of the intermolecular hydrogen bonding formed between -OH and $-NH_2$ groups among CH has been broken. Solubility tests showed that CHAA formed viscous and transparent gel in distilled water.

3.2. Characterization of chitosan ascorbate

The FTIR spectra of the CH and CHAA are shown in Fig. 2, a broad band at 3350 cm^{-1} is typically attributed to the stretching vibration of $-NH_2$ groups, -OH groups, and intermolecular hydrogen bonds. Peak at 1150 cm^{-1} can be attributed to the asymmetric stretching of the C–O–C bridge, and this two bands are characteristic of chitosan[24, 25].

When AA was added into the chitosan-water suspension, chitosan dissolved rapidly. The AA was oxidized to *L*-dehydroascorbic acid (DHA) in water solution and may react with the amine groups on chitosan molecules through Schiff base reaction[26]. The infrared spectra of CHAA therefore confirm that chitosan reacts with DHA because after modification, an additional band appears at 1720 cm^{-1}, due to the presence of carbonyl groups. There is no absorption band at around 1760 cm^{-1}, implying the absence of α-β unsaturated cyclic ketones[15], also the differences of intensity of the carbonyl group at the 1650 cm^{-1} peak and the increase C=O bond of the acetamide groups at 1645 cm^{-1} is due to the loss of the amine groups in the modification of CH to form CHAA.

3.3. DPPH radical scavenging activity of chitosan ascorbate

The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating the free radical-scavenging activities of antioxidants[27]. DPPH radical scavenging activity of CHAA was higher that CH, but similar than that of ascorbic acid (Fig. 3). The highest DPPH radical scavenging activities were 93.70%, 93.96% and 35.47% for CHAA, AA, and CH, respectively at the concentration of 1 mg/mL. CHAA showed stronger DPPH radical scavenging activity than CH at the range of 0.2-1 mg/mL.

3.4. Inhibition of lipid peroxidation of chitosan ascorbate

Lipid peroxidation is a common consequence of free radical mediated chain reactions and some of its end-products that can damage DNA directly or indirectly[28]. The effect of CHAA on peroxidation was shown in Fig. 4. In the linoleic acid lipid peroxidation system, CHAA, CH and AA inhibited lipid peroxidation. The inhibitory effect of lipid peroxidation by CHAA, CH and AA were 80.47%, 35.47% and 81.55% at a concentration of 0.2 mg/mL and 89.99%, 71.66% and 91.18% at a concentration of 1 mg/mL, respectively , indicating that CHAA possessed high lipid peroxidation inhibiting that chitosan at the range of 0.2-1 mg/mL.

3.5. Nitric oxide radical scavenging activity

Nitric oxide (NO), being a potent pleiotropic mediator in physiological processes and a diffusible free radical in the pathological conditions, reacts with superoxide anion and form a potentially cytotoxic molecule, the peroxynitrite ($ONOO^-$). Its protonated form, peroxynitrous acid (ONOOH), is a very strong oxidant[29]. Scavenging activities of CH and CHAA against NO were presented in Fig. 5. Notably, CHAA showed stronger scavenging activity than CH. However, the scavenging effect of CHAA was weaker than that of ascorbic acid. The scavenging effects of CHAA, CH, and AA increased with the increase of sample concentration ranging from 0.2 to 1 mg/mL. At a concentration of 1 mg/mL, the scavenging activity was 77.29%, 49.7% and 79.75% for CHAA, CH and AA, respectively. The results demonstrated that CHAA showed strong activities than that CH.

3.6. Hydrogen peroxide radical scavenging

Although it is not a free radical, H_2O_2 is very harmful to cells because it may cross biological membranes and the highly reactive hydroxyl radical can be synthesized from it by a Fenton reaction[30]. The inhibition of hydrogen peroxide radical by CH, CHAA and AA at varying concentration was showed in Fig. 6. Scavenging activities of CH against hydrogen peroxide was lower than that of CHAA at the range of 0.2-1 mg/mL. At a concentration of l mg/mL, the scavenging rates of CH, CHAA and ascorbic acid were 56.85%, 72.41% and %, 66.83 %, respectively. Therefore, CHAA showed strong peroxide hydrogen scavenging activity.

3.7. Antibacterial activities

The antibacterial activity of chitosan has been studied and demonstrated by several study, while AA has not been reported to have antibacterial activity. In this study the antibacterial activity of CH and CHAA were compared against Gram-positive and Gram-negative bacteria by micro dilution method, and the results are shown in (Table 1). In all case CH exhibited a MIC values better than that CHAA against all bacteria. In fact the antimicrobial performance of the chitosan needs the positively charged amino groups, which could react with the anionic groups of the microbial cell surface[31], the inhibition of the mRNA and protein synthesis and the formation of an external barrier, chelating metals and provoking the suppression of essential nutrients to microbial growth[32, 33], and the modification process compromised the antibacterial activity of chitosan to some degree. Since the amine groups of chitosan are responsible for the antibacterial activity, the reduced activity can be attributed to the consumption of some of the amine groups in the modification process. However, this effect is compensated by the additional antioxidant properties and the CHAA still showed antibacterial ability. Wounds are usually accompanied by microbial infections and free radicals accumulation which would delay the wound healing process[34]. Thus, the confirmed antibacterial ability of CHAA, as well as its antioxidant property, would be beneficial the wound healing process.

CONCLUSION

Combining antimicrobial and antioxidant activities developed from chitosan and ascorbic acid can be expected to have good potential for biomedical applications. In this study, CHAA was synthesized via the Schiff base reaction. The antioxidant and antibacterial activity of CHAA was compared with CH. In all case CHAA exhibited a strong antioxidant activity and better than that CH. The minimum inhibitory concentrations of CH and CHAA against bacteria (*E. coli, P. aeruginosa, S. Typhimurium, L. monocytogenes, S. aureus*) were determined; and the antibacterial efficacy of CH is higher than

that of CHAA because of the consumption of some amine groups on chitosan during the reaction process. However, CHAA still possesses antibacterial efficacy against both bacteria. The decrease in antibacterial activity is compensated by additional antioxidant ability. On the basis of the results obtained, CHAA with presumed antioxidant and antibacterial properties may be used as a possible food supplement or ingredient or in the medical and pharmaceutical industry.

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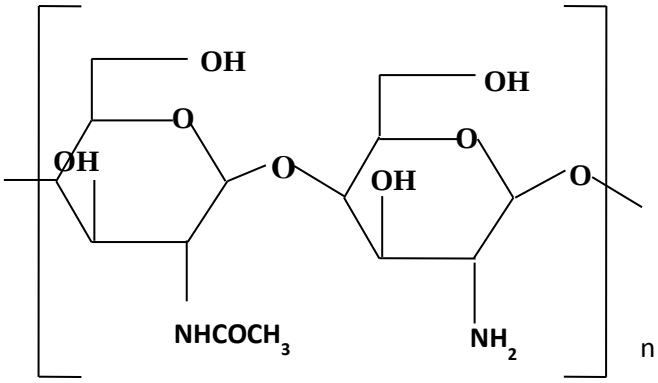
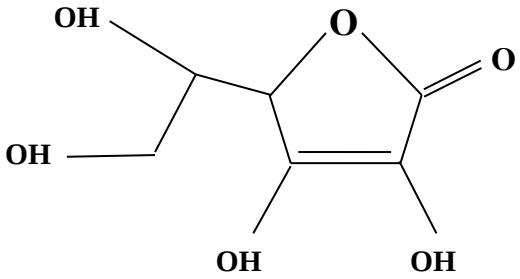
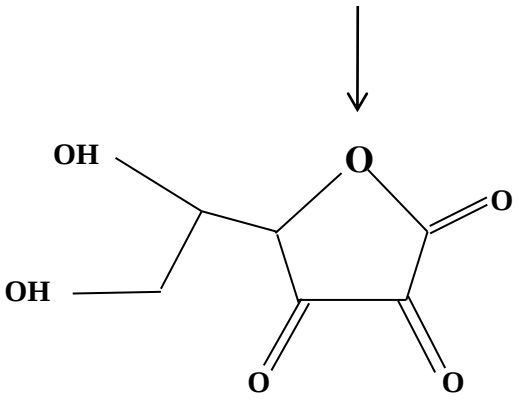


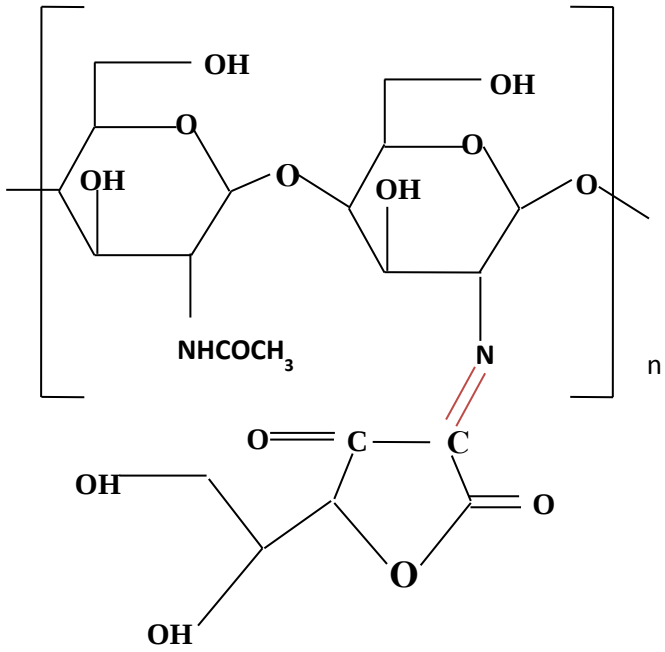
Chitosan



***L*-ascorbic acid**



***L*-dehydroascorbic acid**



Chitosan ascorbate

Fig. 1: A schematic representation of chitosan ascorbate

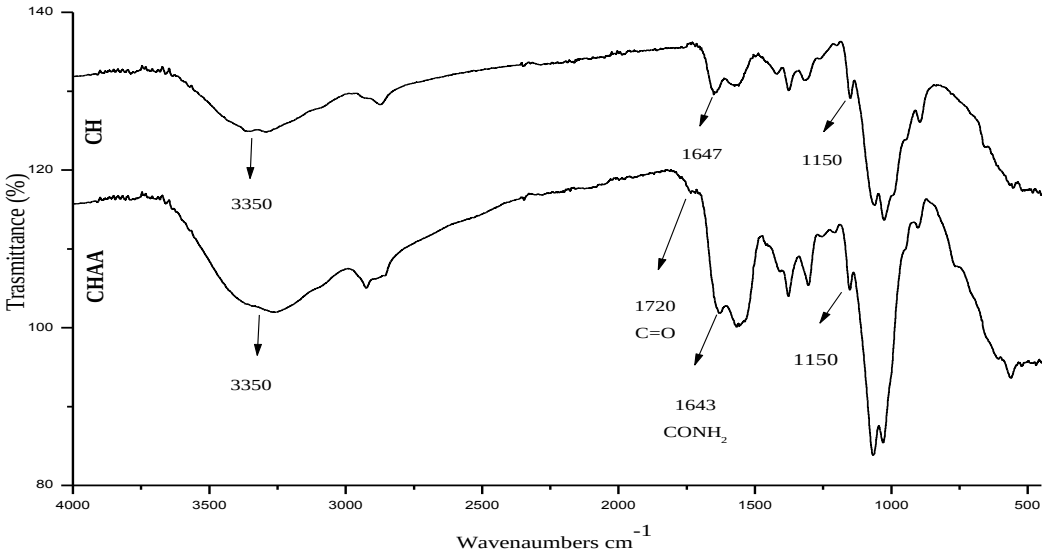


Fig. 2: FTIR spectrum of chitosan (CH) and chitosan ascorbate (CHAA)

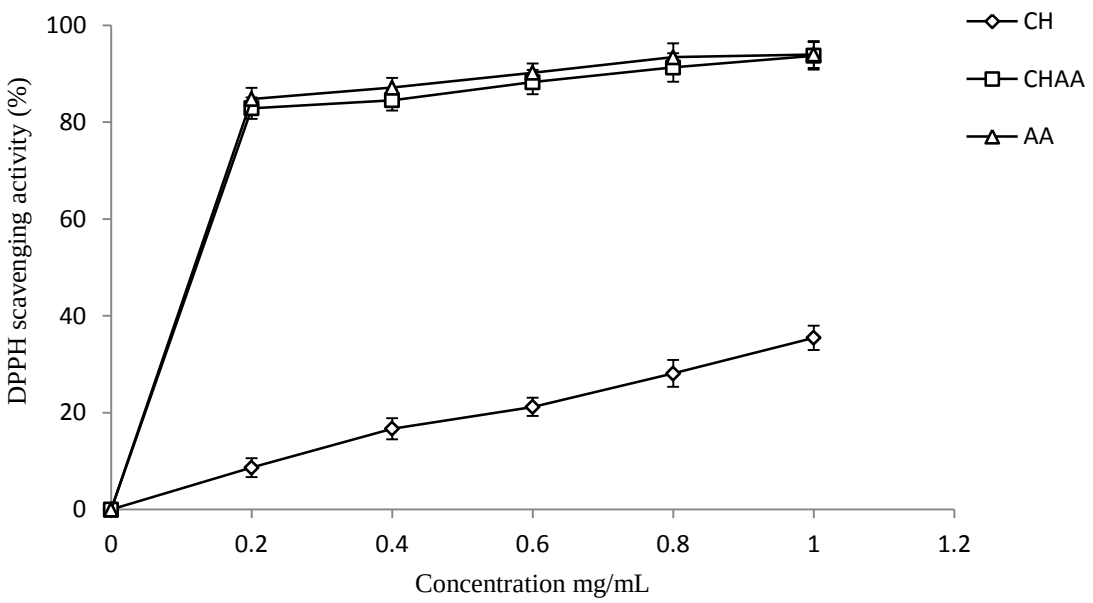


Fig. 3: DPPH radical scavenging activity of CH (chitosan), CHAA (chitosan ascorbate) and AA (ascorbic acid). Each value is presented as mean ± SD (n = 3)

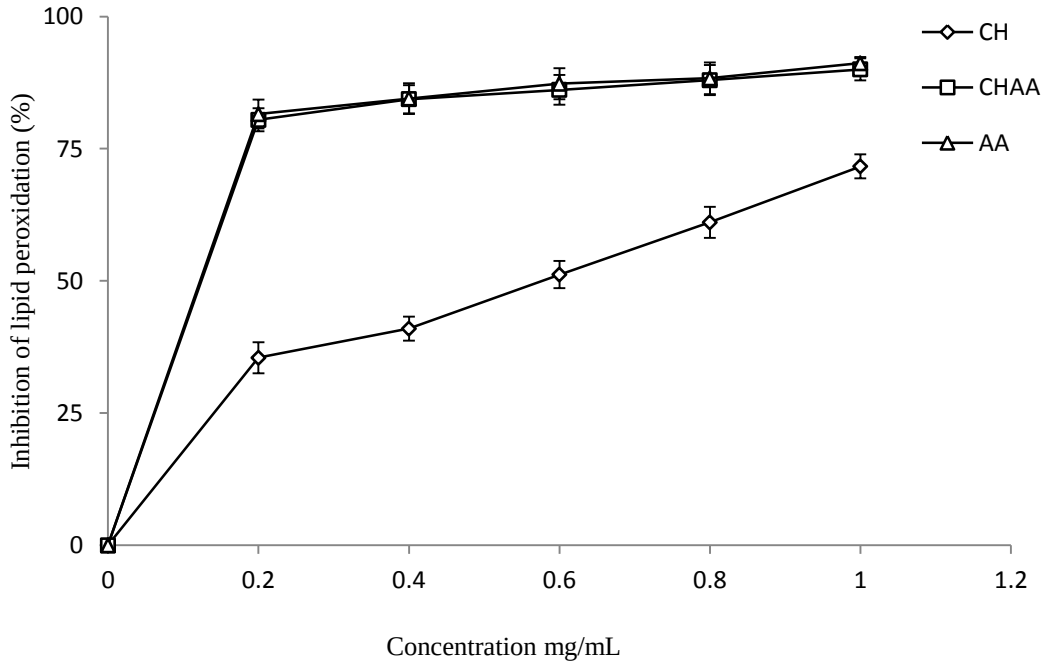


Fig. 4: Inhibition of lipid peroxidation by CH (chitosan), CHAA (chitosan ascorbate) and AA (ascorbic acid). Each value is presented as mean ± SD (n = 3)

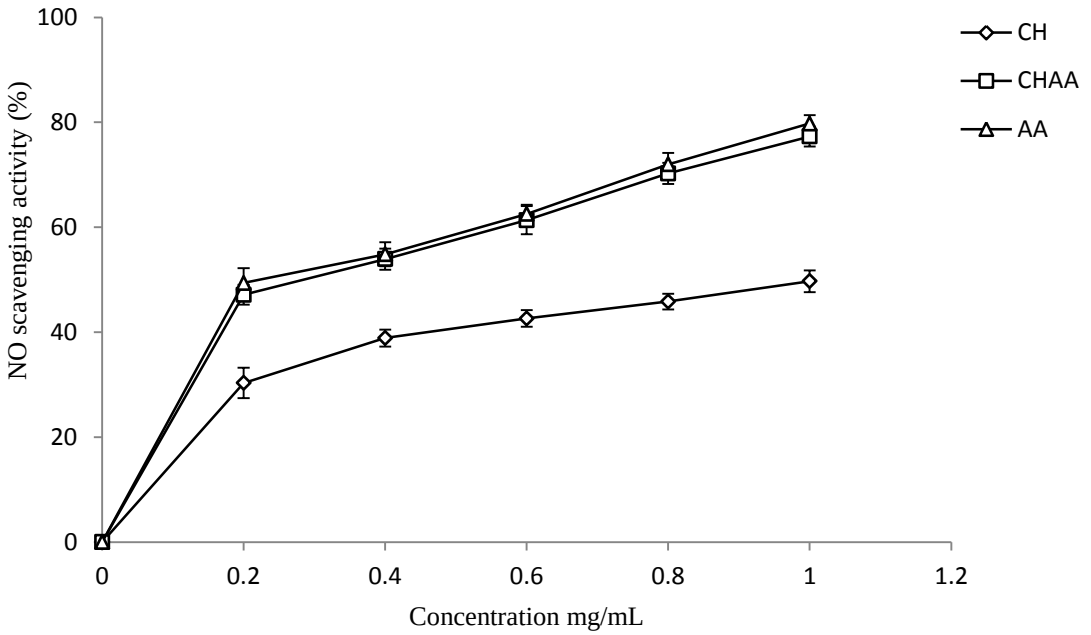


Fig. 5: Nitric Oxide radical scavenging activity of CH (chitosan), CHAA (chitosan ascorbate) and AA (ascorbic acid). Each value is presented as mean ± SD (n = 3)

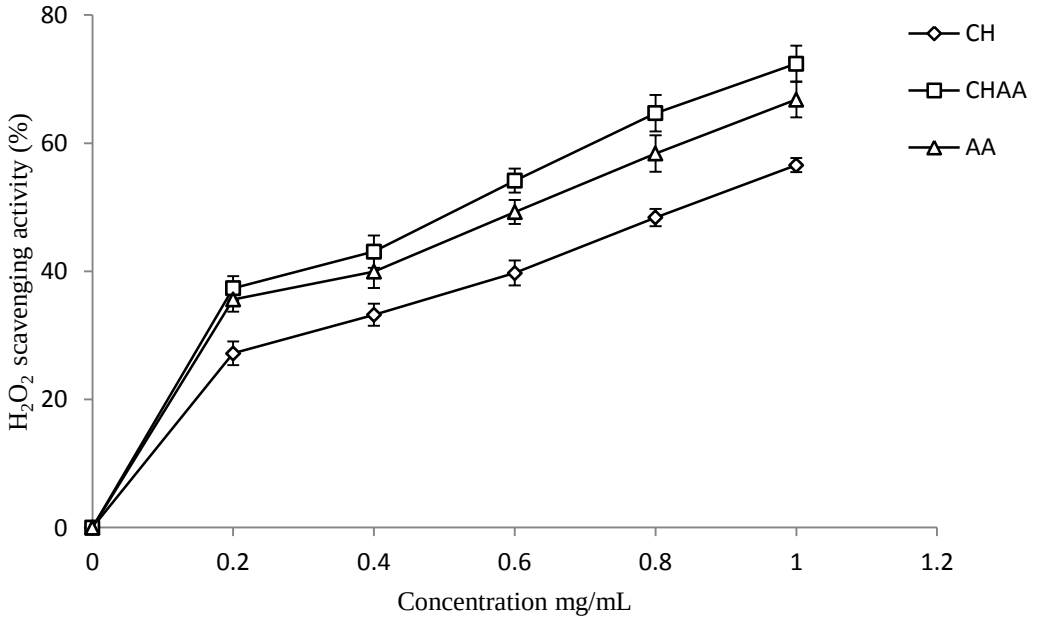


Fig. 6: Hydrogen peroxide radical scavenging activity of CH (chitosan), CHAA (chitosan ascorbate) and AA (ascorbic acid). Each value is presented as mean ± SD (n = 3)

Table 1: MIC of CH and CHAA against bacteria

Test Organisms	MIC values (mg/mL)				
	S. aureus	*E. coli*	*L. Monocytogenes*	*P. aeruginosa*	*S. typhimurium*
CH	2.5	1.25	1.25	0.625	1.25
CHAA	>5	5	2.5	2.5	5

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