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In vitro antioxidant activity of chitosan and chitosan mediated silver nanoparticles

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ABSTRACT
In the present study, chitosan. a non-toxic, biodegradable and biocompatible polymer, is extracted from crab shell wastes (Portunus pelagicus) by deacetylation process. By one step green synthesis process, chitosan mediated silver nanoparticles (CH-AgNPs) were synthesized. The antioxidant activities of chitosan and CH-AgNPs were evaluated through in vitro hydroxyl, H_2O_2 and NO radical scavenging activities at different concentrations (50, 100, 150, 200 mg/mL). CH-AgNPs showed higher Hydroxyl, H_2O_2 and NO radical scavenging activity than chitosan due to the synergistic effect of silver nanoparticles mediated by chitosan. The characteristics of the biosynthesized CH-AgNPs suggest their application as a potential antioxidant agent in removing ROS species produced during oxidative stress inside the body.
Keywords: *Crab shell wastes, Deacetylation, Chitosan, CH-AgNPs, radical scavenging*

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INTRODUCTION

Chitin is the second most abundant polymer present as structural component in the exoskeleton of crustaceans such as crabs, shrimps, lobsters, and squid pens, in insect's cuticle and also in the wall of fungi [1]. But it is insoluble in most of the solvents, because of its compact structure. Therefore, the chemical modification of chitin is performed to make it into a useful product to be used in various fields. Chitosan (β-1,4-linked 2-amino-2-deoxy-D-glucose) is a linear, non-toxic polymer made up of glucosamine and N-acetylglucosamine subunits obtained from chitin by partial deacetylation process. When the degree of deacetylation (DDA) reaches higher than 50%, chitosan becomes soluble in acidic aqueous solutions and it behaves as a cationic polymer [2]. It was the only natural cationic polymer, which allows forming complexes with negatively charged other polymers [3]. Over the past several years, natural polymers, especially chitosan, has received increased attention as one of the most promising renewable polymer for its extensive application in the pharmaceutical and biomedical industries. It has many biological properties like anti-bacterial, anti-fungal, antioxidant, non-toxicity, biocompatibility and biodegradability [4,5,6] that make them attractive for a wide variety of medical applications. In recent years, green synthesis of nanoparticles has been developed rapidly. Green synthesized silver nanoparticles (AgNPs) are increasingly becoming prevalent in all areas of research due to its unique physical properties, cost effectiveness, ecofriendly nature and non-toxic to eukaryotic cells [7]. Phytocompounds present in the plants will enhance the antibacterial and antioxidant properties of AgNPs. Oxidative stress is a difference between free radicals and antioxidants in our body. Free radicals are compounds that produce oxygen, with an unequal number of electrons which helps them to react to other molecules easily. Oxygen derived free radicals are called as Reactive oxygen species (ROS) such as superoxide radical (O2), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) generated continuously during various metabolic processes [8].

Reactive oxygen species (ROS) plays a crucial function in the pathophysiology of many chronic diseases and therapy using antioxidants plays a major role in providing effective protection against them. Synthetic antioxidants like BHT (Butylated hydroxytoluene) and BHA (Butylated hydroxyanisole) are

known to be effective free radical scavengers but may have side effects [9]. The quest for natural derived antioxidants as alternatives to synthetic antioxidants is therefore of considerable significance. Chitosan polymer being non-toxic, biocompatible and biodegradable, researchers has paid attention to evaluate the antioxidant activity of chitosan [10,11,12] and its derivatives [13,14]. Furthermore, antioxidant properties of silver nanoparticles mediated by plant extracts have also been demonstrated [15,16]. Similarly, antimicrobial and antioxidant efficacy of AgNPs with commercially obtained chitosan was evaluated [17,18]. The present study was conducted to demonstrate the *in vitro* antioxidant properties of chitosan extracted from *P. pelagicus* shells and chitosan mediated silver nanoparticles (CH-AgNPs).

MATERIALS AND METHODS

Chitosan – Stock and Working solution preparation

0.5g of chitosan was dissolved in 100 ml of 1% acetic acid solution which was used as stock solution from which the desired working concentrations (50 µg/ml, 100 µg/ml and 200 µg/ml) were prepared through appropriate dilutions.

CH-AgNPs - Stock and Working solution preparation

0.5 g of CH-AgNPs was dissolved in 50 ml of 1% acetic acid solution which was used as stock solution from which the desired working concentrations (50 µg/ml, 100 µg/ml and 200 µg/ml) were prepared.

In vitro antioxidant studies:

Hydroxyl radical scavenging assay:

Fenton reaction between the deoxyribose and the test samples (chitosan and CH-AgNPs) were measured for the hydroxyl radical scavenging activity [19]. Reaction mixture contains $FeCl_2$, 1,10-phenanthroline, phosphate buffer, H_2O_2 and test sample were incubated for 5min at room temperature. Based on their degradation ability, the hydroxyl groups were generated and forms pink colored chromogen. Absorbance was measured at 560 nm with a spectrophotometer. The analysis was performed in triplicates. The percentage of hydroxyl radical scavenging activity by the chitosan and CH-AgNPs was calculated as follows:

$$\% \text{ Inhibition [Hydroxyl radical]} = \frac{Absorbance\ sample - Absorbance\ control}{Absorbance\ control}\ x\ 100$$

Hydrogen peroxide radical scavenging activities:

The ability of chitosan and chitosan mediated silver nanoparticles (CH-AgNPs) to scavenge hydrogen peroxide radical was determined according to standard procedures [20]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer. Different concentrations of both the chitosan and CH-AgNPs (50, 100, 150, 200µg/ml) were prepared and added to hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The analysis was performed in triplicates. The percentage of hydrogen peroxide scavenged by the chitosan and CH-AgNPs was calculated as follows:

$$\% \text{ Inhibition } [H_2O_2] = \frac{Absorbance\ sample - Absorbance\ control}{Absorbance\ control}\ x\ 100$$

Nitric oxide scavenging activities:

The nitric oxide (NO) scavenging activity of chitosan and chitosan mediated silver nanoparticles (CH-AgNPs) was determined by Griess reaction [21]. Sodium nitroprusside of 100µl was prepared in phosphate buffer with different concentrations (50, 100, 150, 200µg/ml) of chitosan and (CH-AgNPs) for 60 min, at room temperature under light. After incubation, 100 µL of Griess reagent was added to each well. The mixture was incubated at room temperature for 10 min and the absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylehylendiamine was read at 562 nm. The analysis was performed in triplicates. The percentage of NO scavenged by the chitosan and CH-AgNPs was calculated as follows:

$$\% \text{ Inhibition [NO]} = \frac{Absorbance\ sample - Absorbance\ control}{Absorbance\ control}\ x\ 100$$

Chitosan and CH-AgNPs providing 50% inhibition (IC_{50}) under each assay condition was calculated from the graph of inhibition percentage against sample concentration.

Statistical analysis

All experiments were carried out in triplicates, and average values with standard deviation were reported with the help of SPSS software (17.0 version). Duncan post hoc testing was performed between the samples mean comparison.

RESULTS

Hydroxyl radical scavenging activity

The scavenging effect of chitosan and CH-AgNPs of hydroxyl radicals was shown in Table. 1. Inhibition efficiency of chitosan ranged from 45.98–72.98% whereas CH-AgNPs efficiency ranged from 58.03–82.88% (Fig. 1) in all the tested concentrations. The scavenging activity was found to be increased with the increase in concentration. The IC 50 value of chitosan was 44.4 µg/ml and CH-AgNPs was 44 µg/ml.

Hydrogen peroxide scavenging activity

The results of H_2O_2 scavenging activity of chitosan and CH-AgNPs was shown in Table 2. The scavenging activity was determined to be increased with the increase in the concentration of chitosan and CH-AgNPs from 50 to 200 µg/ml. The percentage of inhibition of the hydroxyl radical was varying from 45.98–72.98% (Fig. 2). CH-AgNPs showed higher inhibition than chitosan varying from 55.01–82%. The IC50 value chitosan and CH-AgNPs were 46.03 µg/ml and 44.99 µg/ml respectively.

Nitrogen oxide scavenging activity

The nitrogen oxide scavenging activity at different concentrations of chitosan and CH-AgNPs were presented in Table 3. The NO scavenging assay revealed CH-AgNPs showed pronounced scavenging ability when compared to chitosan (Fig. 3). Maximum inhibition was observed at 200µg/ml chitosan and CH-AgNPs concentration as 72.0% and 82.88% respectively.

DISCUSSION

The body generates free radicals of reactive oxygen species (ROS) and reactive nitrogen (RNS) species by specific endogenous mechanisms, when exposed to numerous physiochemical factors or infectious conditions. These free radicals may cause damage to DNA, alter the growth signals in cells and change the gene expression. This phenomena leads to many diseased condition like cancer, diabetes, cardiovascular diseases and atherosclerosis [22]. Therefore it is very essential to maintain steady balance between the free radicals and antioxidants in optimal level inside our body. Many compounds have been suggested to possess antioxidant properties which may help to inhibit lipid peroxidation and free radical mediated oxidative stress [23]. Although our body is well-equipped with antioxidant defense system to protect from oxidative damage, such systems are inadequate with endogenous antioxidants produced in our body. Consequently, endogenous antioxidants should be added in our diet which will help to reduce oxidative damage to the body. Nitric oxide (NO) is a highly reactive free radical material, an essential intracellular and intercellular signaling agent used to control a number of physiological and pathophysiological pathways such as cardiovascular, nervous, and immune systems [24,25].

DPPH assay is the commonly used approach for determining the antioxidant potential of natural compounds. It is an inexpensive and spectrophotometric technique based on quenching of stable colored radical [26]. This free radical changes from violet to yellow colour upon reduction by donating an electron. The results of the present investigation reveal that the chitosan and CH-AgNPs possess a property to inhibit DPPH free radical formation and scavenging activity. Also the hydroxyl radical, (OH•) is the neutral form of the hydroxide ion ($OH^•$). It is a short lived, highly reactive ROS species produced from the decomposition of hydroperoxides (ROOH) or by oxidation of water or hydroxyl ions when reacts with excited atomic oxygen with water [27]. Addition of chitosan and CH-AgNPs to the reaction mixture efficiently removed the ($OH^•$) radicals and prevented the degradation of deoxyribose in a dose dependent manner. Hydrogen peroxide is generated inside the body by the dismutation of superoxide radical (O_2•) catalyzed by superoxide dismutase enzymes. Also it is produced directly by the peroxisomal pathway for β-oxidation of fatty acids. H_2O_2 levels in human urine may be used as biomarker of oxidative stress [28]. It is less reactive, but highly cytotoxic and must be immediately eliminated from the body to control its deleterious effects. The present study revealed the potent H_2O_2 radical scavenging activity for the chitosan and CH-AgNPs. Nitric oxide ($NO^•$) is also a highly reactive free radical formed during its reaction with oxygen or superoxide ions. It mediates a wide range of physiological processes such as cardiovascular, nervous, and immune systems [29]. Here, the nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. Study with this radical revealed that both the chitosan and CH-AgNPs are potent scavengers of nitric oxide free radical. The antioxidant activity of chitosan is mainly due to the amine group at the C2 position and hydroxyl group at the C6 position [30,31]. According to [32] hydroxyl radical scavenging activity of chitosan is attributed to its metal chelating ability. But CH-AgNPs showed better inhibitory activity than chitosan in all the concentrations due to its high synergistic effect. Chitosan ascorbate showing strong antioxidant activities than chitosan has been demonstrated [33]. Also, silver/chitosan composites showed high free radical scavenging activities than chitosan has been reported [18].

Table 1. Inhibition (%) of hydroxyl scavenging activities of chitosan and CH-AgNPs

Concentrations (µg/ml)	Inhibition (%)	
	Chitosan	CH-AgNPs
50	50.98±0.40	58.03±0.30
100	58.95±0.59	65.60±0.35
150	68.55±0.28	75.68±0.58
200	72.00±0.39	82.88±0.59

Values are the mean ± SD, n=3.

Table 2. Inhibition (%) of hydrogen peroxide scavenging activities of chitosan and CH-AgNPs

Concentrations (µg/ml)	Inhibition (%)	
	Chitosan	CH-AgNPs
50	45.98±0.35	55.01±0.49
100	52.13±0.29	63.98±0.70
150	61.03±0.30	75.90±0.55
200	72.98±0.32	82.0±0.43

Values are the mean ± SD, n=3.

Table 3. Inhibition (%) of Nitric oxide scavenging activities of chitosan and CH-AgNPs

Concentrations (µg/ml)	Inhibition (%)	
	Chitosan	CH-AgNPs
50	55.06±0.29	64.0±0.20
100	60.81±0.51	69.21±0.34
150	64.16±0.38	76.13±0.55
200	75.13±0.36	81.1±0.28

Values are the mean ± SD, n=3.

Fig1. Hydroxyl scavenging activities of chitosan and CH-AgNPs

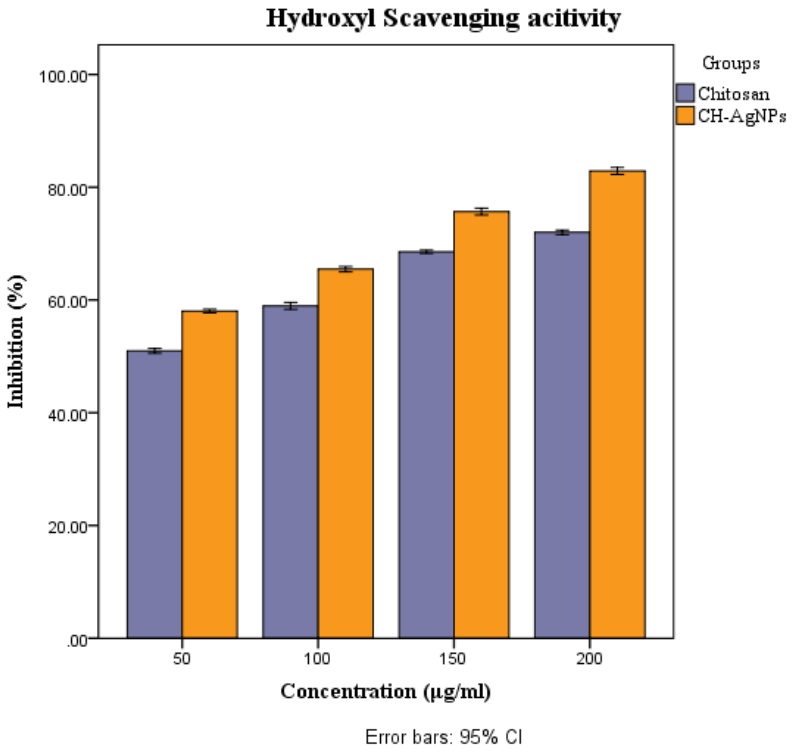


Fig 2. Hydrogen peroxide scavenging activities of chitosan and CH-AgNPs

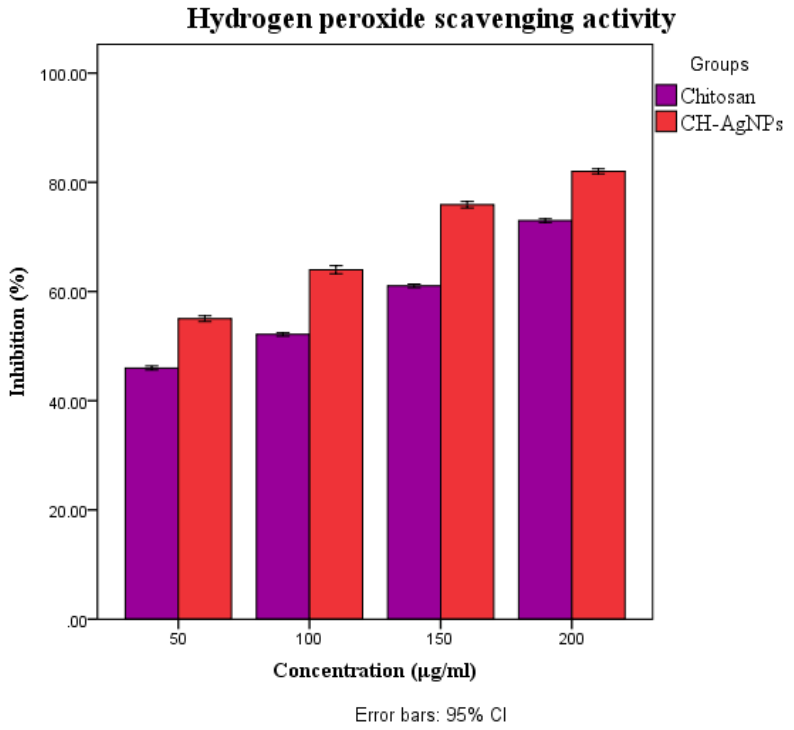
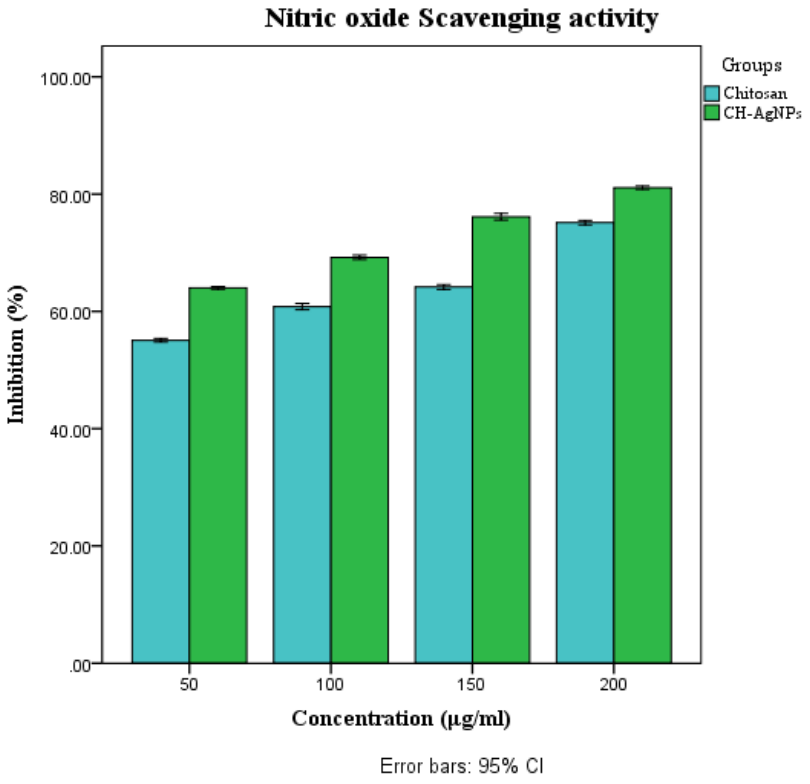


Fig 3. Nitric oxide scavenging activities of chitosan and CH-AgNPs



CONCLUSION

Hydroxyl, H_2O_2 and NO scavenging activities for chitosan and CH-AgNPs were analysed. CH-AgNPs showed significantly increased antioxidant properties than chitosan in all the concentrations. This increased activity was due to the enhanced chelating properties of CH-AgNPs, that actively involved in ROS scavenging. In comparison to chitosan, CH-AgNPs showed enhanced activity due to larger surface

area to volume ratio. From the above results, we conclude that CH-AgNPs showed good antioxidant activity against reactive oxygen species generated during oxidative stress. CH-AgNPs with presumed antioxidant properties may be used as alternative to synthetic antioxidants in food, medical and pharmaceutical industry.

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CONFLICTS OF INTEREST
The authors declare that they have no conflicts of interest.

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