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Antimicrobial and Antioxidant Properties of *Ceriodaphnia quadrangula* Ephippia Chitosan

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MELTEM ASAN-OZUSAGLAM[1,2]*, YAVUZ SELİM CAKMAK[1,2], MURAT KAYA[1,2], SEVİL ERDOGAN[3], TALAT BARAN[2,4], AYFER MENTES[2,4], IHSAN SAMAN[1,2]
[1]Department of Biotechnology and Molecular Biology, Faculty of Science and Letters, Aksaray University, 68100, Aksaray, Turkey; [2]Science and Technology Application and Research Centre, Aksaray University, 68100, Aksaray, Turkey; [3]Keşan Vocational College, Fisheries Programme, Trakya University, 22800 Keşan, Edirne, Turkey; [4]Department of Chemistry, Faculty of Science and Letters, Aksaray University, Aksaray, Turkey
***Address correspondence to:**: meltemozusaglam@gmail.com*

Abstract

The chitosan from ephippia of Ceriodaphnia quadrangula (Crustaceae) has been fully characterized in our previous study, and in this work it was tested for antibacterial and antifungal activities against human and fish pathogens, while also determining its antioxidant activities. Using the disc diffusion method showed that the antimicrobial activity was observed against all tested microorganisms in the range of 12.84 – 16.28 mm.The minimal bactericidal concentration (MBC) value was found to be 0.63 - 2.50 mg/ml. Chitosan obtained from C. quadrangula ephippia showed higher antimicrobial activity against some pathogenic bacteria than commercial antibiotics. While chitosan inhibited 35.83% of the 1,1-diphenyl-2-picrylhydrazyl radicals (at 5 mg/ml), it showed an activity of 14.48 µg/ml for ferrous ion reducing. Consequently, it is suggested that chitosan obtained from the ephippia of C. quadrangula (Crustacea) can be used as food/feed additives, preservatives or in the pharmaceutical industry instead of using synthetic antimicrobials and antioxidants.

Keywords: Chitosan, human pathogens, fish pathogens, antibacterial, antifungal, DPPH, ferric ion reducing power

1. Introduction

Nowadays, concerns in relation to the harmful effects of synthetic antimicrobial products and antioxidants are increasing due to an increase in consumer awareness. In the last thirty years, the problem of developing resistance against antibiotics has emerged. As a result of the resistance developed against synthetic antibiotics used against diseases, there has been a decrease in the effectiveness of these antibiotics. In addition, these kinds of synthetic antimicrobials can sometimes cause important problems, such as hypersensitivity, allergic reactions and immunity suppression in humans [1]. These kinds of synthetic antimicrobial agents used in animal feed can cause health problems both in animals and the people who consume them, while they also pollute the ecosystem [2]. Customers' preferences have changed from synthetic antioxidants to natural ones, because synthetic phenolic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), used in cosmetics and therapeutics are highly volatile and non-stable at high temperatures [3]. There are also strict regulations on the use of synthetic food additives, and some synthetic antioxidants have carcinogenic properties [3]. For these reasons, nowadays biotech companies are focused on developing alternative natural products instead of synthetic products [4]. Recently, the antioxidant activities of chitosan and its derivatives, which are natural and alternative to synthetic products, have gained attention [5]. As a result, the use of natural antimicrobial and antioxidant agents, like chitosan, has been gaining importance particularly

in the food/feed and health industries in recent years. Chitin is a polysaccharide found in the exoskeleton of organisms such as Crustacea and Insecta in the phylum Arthropoda, in the cell walls of fungi and in the bodies of Spongea and Nematoda [6]. Chitosan is a commercial biopolymer obtained from the deacetylation of chitin with sodium hydroxide (NaOH) at a high concentration [7]. While chitin is considered a crude product due to its insolubility in organic solvents, chitosan is widely used in industry because it is soluble in weak acids. Chitosan and its derivatives are used in many different areas, such as agriculture (fungicide, film, soil modifier, elicitor), waste water treatment (precipitant agent), food industry (coating, preservative, antimicrobial and antioxidant agent), cosmetics (lotion, in facial and body creams) and nowadays in the biomedical field as a pharmaceutical agent (fibers, artificial organs, drugs, membranes, anti-cancerogenic agents, substances used in accelerating wound healing) [8, 9, 10, 11, 12, 13, 14]. Chitosan is a biomaterial that is highly preferred by researchers because of its properties, such as biocompatibility, biodegradability, antitumor, antimicrobial and antioxidant activities. Due to the numerous properties of chitosan, antimicrobial and antioxidant activities were investigated in this study. In studies performed so far, the antimicrobial and antioxidant activities of chitosans from fungi [15, 16, 17], crab shell [18, 19], shrimp shells [20, 21, 22, 23] or commercial chitosans [24, 25, 26, 27] have been tested. In our study, the antibacterial and antifungal activities against various human and fish pathogens as well as the antioxidant effect of the fully characterized chitosan [28] which was obtained from water flea eggs (*Ceriodaphnia quadrangula* ephippia) were investigated.

2. Materials and Method

2.1. Chitosan production

Chitosans previously obtained and fully characterized by Kaya et al. [28] were used in this study to determine their antimicrobial and antioxidant activities. For more information about *C. quadrangula* see, Kaya et al. [28].

2.2. Preparation of chitosan stock solution

Chitosan from ephippia of *C. quadrangula* was dissolved in 1% acetic acid at a concentration of 5 mg/ml. After stirring, the solution was sterilized by autoclaving at 121ºC for 20 min and stored at 4ºC for subsequent use.

2.3. Microbial strains and culture conditions

In vitro antimicrobial studies against clinical and food-borne pathogens were carried out on two Gram-positive bacteria (*Bacillus subtilis* RSKK 244 and *Listeria monocytogenes* ATCC 7644), two Gram-negative bacteria (*Yersinia enterocolitica* NCTC 11175 and *Salmonella enteritidis* RSKK 171), and one yeast (*Candida albicans* ATCC 10231). The following fish pathogen bacteria were also used in the screening of antibacterial activity: two Gram-positive bacteria (*Lactococcus garvieae* and *Streptococcus agalactiae* Pasteur Inst. 55118) and one Gram-negative bacterium (*Vibrio alginolyticus*).

2.4. Inhibitory effect determined with the disc diffusion method

The antimicrobial activity was determined with disc diffusion method [29]. One hundred microlitres of suspension (0.5 McFarland turbidity) of the test microorganisms were spread on agar plates. Filter paper discs (6 mm in diameter) were placed on the inoculated plates and then impregnated with 50 µl (250 µg/disc) of the chitosan solution. Antibiotic discs of Ampicillin (Amp, 10 µg/disc), Gentamicin (CN, 10 µg/disc), Kanamycin (K, 30 µg/disc), Amikacin (AK, 30 µg/disc), Erythromycin (E, 15 µg/disc) and Fluconazole (FCA, 25 µg/disc) were also used as positive controls. 1% acetic acid was used as the negative control. The diameters of the inhibition zones (mm), including disc diameter, were measured.

2.5. Minimal bactericidal (MBC)/fungicidal (MFC) concentration

The micro-dilution method using serially dilutions (2 fold) was used to determine the MBC or MFC values of the chitosan solution according to Chandrasekaran and Venkatesalu [30] with some modifications [31]. For the MBC or MFC values, the concentrations of the chitosan solutions that showing no growth of a microorganism on the agar plates were recorded.

2.6. Ferric ion reducing power

In this method, chitosan solutions at the concentrations of 5000 µg/ml and 625 µg/ml were used. BHT also was prepared at five concentrations between 200 µg/ml and 3.125 µg/ml as a standard. 2.5 ml from the chitosan solutions at different concentrations was taken. 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were added to the tube. Tubes were incubated at 50°C for 20 minutes. After incubation, 2.5 ml of 10% TCA was added to the tubes. After stirring thoroughly, 2.5 ml of the mixture from the upper portions of the tube was transferred into another tube. Then 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ solution were added into the new tube. The absorbance of the solutions was measured at 700 nm [32]. Ferric ion reducing power also increased depending on the increase in absorbance. The EC_{50} value whose absorbance was 0.5, which means it was an effective concentration, was calculated using a concentration absorbance graphic. When the EC_{50} value gets lower, the antioxidant activity increases.

2.7. Free radical scavenging activity (DPPH, 2,2-diphenyl-1-picrylhydrazyl)

Chitosan solutions were prepared at four concentrations between 5000-625 µg/ml. BHT, which is a synthetic antioxidant, was prepared at five concentrations between 200 µg/ml and 3.125 µg/ml. The DPPH solution was prepared at a concentration of $6x10^{-5}$ M and five concentrations were obtained for a calibration curve by diluting from this stock. 0.5 ml of the chitosan solutions at different concentrations were taken and 3 ml of the DPPH solutions at the concentration of $6x10^{-5}$ M were added to them. Tubes were kept in the dark at room temperature for 30 minutes after closing firmly and stirring vigorously. After this time, absorbances were measured at 517 nm. The inhibitions of chitosan solutions and BHT were calculated according to the following equation:

$$\text{Inhibition}(\%)=((A_{control}-A_{sample})/A_{control})x100$$

A concentration-inhibition graphic for the chitosan extracts and BHT was constructed and the IC_{50} value was calculated. A low IC_{50} value shows that the antioxidant capacity is high [33].

3. Results and Discussion

3.1. Antimicrobial activity

In the present study, the antimicrobial activity of chitosan from the ephippia of *C. quadrangula* was determined on the basis of disc diffusion and micro-dilution assays. Antibacterial and antifungal activities of the chitosan were screened against four human pathogenic bacterial strains and one fungal pathogen and three fish bacterial pathogenic bacteria as presented in Table 1. Acetic acid (1%) was used as the control and it showed no antimicrobial activity against any of the test microorganisms. The activity of chitosan against human pathogenic bacteria revealed that the highest antibacterial effect was observed against *B. subtilis* RSKK 244 (19.01 mm), which has the largest inhibition zone, and this was followed by *S. enteritidis* RSKK 171 and *Y. enterocolitica* NCTC 11175. On the other hand, within all the tested bacteria, the lowest inhibition zone was determined against *L. monocytogenes* ATCC 7644 (13.38 mm). The chitosan (250 µg/disc) showed better antibacterial activity against *S. enteritidis* RSKK 171 in comparison to the standard

Gentamicin (10 µg/disc), Amikacin (30 µg/disc), Erythromycin (15 µg/disc) and Kanamycin (30 µg/disc). MBC values were determined as 0.63-1.25 mg/ml. *C. albicans* ATCC 10231, among all the tested human pathogenic microorganisms, showed the lowest inhibition zone (12.84 mm) and the highest MFC value (2.50 mg/ml). Appropriate doses of chitosan from *C. quadrangula* could control *C. albicans* derived from a patient with bronchomycosis (ATCC 10231), and could be used as a safe alternative agent to synthetic antifungal products. In a previous study, Tayel et al. [34] determined the anticandidal activities of four fungal chitosans from *Mucor rouxii* DSM-1191 on three *C. albicans* strains. CTS 1 (Chitosan 1) was found to be the most active type to prevent the growth of all *C. albicans* strains. The MICs (minimal inhibitory concentrations) from CTS 1 were 2.00, 1.75 and 1.25 mg/ml against *C. albicans*-A, -H and -C, respectively. One of the most important food-borne pathogens is *L. monocytogenes* [35] which has been isolated from various food products. Vegetable, dairy, meat and poultry products have all been responsible for listeriosis [36, 37, 38, 39, 40]. Listeriosis is known as a potential risk for human health because of its high mortality rate, particularly in immune-suppressed patients, neonates, children, pregnant woman and the elderly [41]. Some *L. monocytogenes* are resistant to some antibiotics including sulphonamide, tetracycline, gentamicin, penicillin, ampicillin, trimethoprim, streptomycin, rifampicin, erythromycin and kanamycin [42]. Consumer concerns about chemical preservatives have generated increased interest about the antimicrobial properties of chitosan applied in food preservation [43, 44]. Bento et al. [16] determined the antimicrobial effect of chitosan from a fungus (*M. rouxxi* UCP064) on *L. monocytogenes* ATCC 7644 to be used as an alternative natural compound. In their study, the MIC and MBC values of the chitosan were found to be 2.5 and 5.0 mg/ml, respectively. In the current study, the MBC value of chitosan from the ephippia of *C. quadrangula* against *L. monocytogenes* ATCC 7644 was lower (0.63 mg/ml) than chitosan from the fungi *M. rouxxi* UCP064. Proper doses of chitosan from *C. quadrangula* can be used as an alternative to commercial and synthetic substances (e.g. antibiotics) and applied in foods. In another study, Islam et al. [45] studied the effect of crab-chitosan on two food borne and clinical pathogens (*Escherichia coli* and *Staphylococcus aureus*). The highest inhibition zones against *S. aureus* and *E. coli* were 13 mm and 10 mm for 800 and 1000 ppm/well concentrations, respectively. The MIC values of the chitosan were found to be 1300 and 1200 ppm for *E. coli* and *S. aureus*, respectively. In a recent study, the antibacterial activity of chitosan was screened against pathogenic clinical isolates and the antibiotic sensitivity of chitosan was compared using standard antibiotic discs. The inhibitory activity of chitosan was found to be greater against *S. aureus* than the Coagulase negative S*taphylococcus* and *Enterococci*. The antibacterial activity of chitosan on *S. aureus* (ATCC 25923) at varying concentrations (100–400 µg) was determined to be higher than standard antibiotics such as Vancomycin and Amoxyclav which are commonly used against these pathogens [46]. Many other studies have been performed to test the antimicrobial activity of chitosans from different sources against many bacteria and fungi. It was revealed in a study by Sukmark et al. [47] that antimicrobial activity of chitosans from different sources was different for each type of bacteria. The results of this study showed while chitosan from crab had the best efficiency for *L. monocytogenes* (MIC: 0.05%), chitosan from squid showed the best antimicrobial activity against *S. aureus*, *B. subtilis* and *B. cereus* (MIC range from 0.05 to 0.06%). In another study, while MIC values of crab shell chitosan varied between 0.025-0.2%, the diameters of inhibition zone were between 9.76-11.92 mm for *L. monocytogenes* [48]. Kaya et al. [31] reported that the MBC values and the inhibition zone diameters of chitosan from *D. longispina* ephippia were betweeen 0.32-1.25 mg/ml and 12.60-17.75 mm, respectively for the same bacterial species used in this study. It was seen

that inhibition zone diameters of *C. quadrangula* chitosan for *B. subtilis* (19.01 mm) and *S. agalactiae* (13.02 mm) was higher than the chitosan of *D. longispina* (17.33 mm and 12.66 mm). Likewise, MBC values and inhibition zone diameters of chitosan from adult and larval Colorado potato beetle were between 0.32-1.25 mg/ml and 13.89-26.03 mm, respectively [49]. Grasshopper chitosan produced from two cosmopolitan orthopteran species (*Calliptamus barbarus* and *Oedaleus decorus*) exhibited a strong antimicrobial activity against tested microorganisms and the MBC or MFC values were determined as 0.16-2.50 mg/ml [50]. For fungi *C. albicans,* MIC values of chitosans from pink shrimp (*Parapenaeus longirostris)* and from *Squilla mantis* were found to be 1.25 mg/ml and 0.156 mg/ml, respectively [51]. The larval chitosan from potato beetle had the lower MFC value (1.25 mg/ml) for *C. albicans* than the adult chitosan (2.50 mg/ml) [49]. In this study, antifungal activity of *C. quadrangula* chitosan against *C.albicans* was similar to that of potato beetle chitosan. Kamala et al. [52] revealed that diameters of inhibition zone of chitosan (at 1 mg/ml concentration) from shrimp (*Parapeneopsis stylifera*) shell waste varied between 3.2-8.9 mm for the tested microorganisms. In this study, the antibacterial activity of chitosan from the ephippia of *C. quadrangula* was also determined against three fish pathogenic bacteria that are commonly encountered in the fisheries sector. They can cause important diseases and mortality in fish [53]. The chitosan exhibited very similar activities against the fish pathogenic bacteria in the range of 13.02-13.88 mm, according to the disc diffusion assay. The highest inhibitory activity of chitosan was against *V. alginolyticus,* which showed a lower MBC value (0.63 mg/ml) in comparison to the other two fish bacterial pathogens (1.25 mg/ml). It was found that chitosan is more effective against *V. alginolyticus* than the traditional antibiotic Ampicillin. White shrimp, *Litopenaeus vannamei,* injected with commercial chitosan at 4 µg/g or less increased its immunity and resistance to *V. alginolyticus* infection [54]. The findings of many studies revealed that the antimicrobial activity of chitosan is dependent upon the physicochemical nature of chitosan, the conditions of the chitosan production process, the testing method and bacterial genus/species tested [48, 55, 56]. Results of our study also supported that the antimicrobial activity was affected by source of chitosan and bacterial species. The data from the this study showed that chitosan from the ephippia of *C. quadrangula* had antibacterial and antifungal activities and could be used as a natural antimicrobial alternative to synthetic agents in the food/feed and pharmaceutical industry.

3.2. Antioxidant Activity

3.2.1. Ferric Ion Reducing Power

The Fe (III) ions' reducing capacity of a solution is frequently used as an indicator of its electron donating ability. Antioxidants usually have their effects determined with this mechanism. For that reason, the capacity of the iron reducing power shows a strong correlation with the results of other antioxidant methods [57]. In the iron reducing power, the absorbance is directly proportional to the antioxidant capacity. The absorbance of the chitosan from the ephippia of *C. quadrangula* was obtained using this method and the ferric ion reducing power is given in Table 2. The concentration-absorbance relationship of chitosan is also given in Figure 1. The lower EC_{50} value indicates a higher antioxidant activity. In the iron reduction method, the EC_{50} values of chitosan from *C. quadrangula* ephippia and BHT, which is a synthetic antioxidant, were found to be 14.48±0.02 µg/ml and 0.04±0.01 µg/ml, respectively. It was reported in a study that the iron reducing power of sulfated chitosan obtained from cuttlefish was an absorbance of 0.300 at the concentration of 0.75 mg/ml [58]. In another study, Kaya et al [49] found EC_{50} values of chitosans from Colarado potato beetle to be 4.53mg/ml for adults

and 4.43mg/ml for larvae. This value was recorded as 10.12 and 8.30 mg/ml for chitosan and O-Carboxymethyl chitosan from *D. longispina* ephippia, respectively [31]. Also it was stated in this study that chitosan from *D. longispina* ephippia showed moderate antioxidant activity. Younes et al. [56] remarked that chitosan from shrimp (*Metapenaeus monoceros*) showed relatively low reducing power. In addition, EC_{50} values was recorded as 7.69-15.71 mg/ml for fungal chitosan by Yen et al. [17] and 13.46–20.00 mg/ml for crab chitosan [19]. Results of all these studies indicated that the ferric ion reducing activity increased depending on the chitosan concentration. Considering these results, we can say that *C. quadrangula* showed a moderate ferric ion reducing activity increasing with the chitosan concentration.

Table 1. Antimicrobial activity of chitosan from *C. quadrangula* ephippia

Test microorganisms	MBC[a] or MFC[b] (mg/ml)	Inhibition zone diameter[c] (mm)	Antibiotics Inhibition zone diameter[c] (mm)					
			Amp	CN	AK	E	K	FCA
L. monocytogenes ATCC 7644	0.63	13.38 ±0.88	25.00 ±0.18	20.77 ±0.13	20.40 ±0.08	39.01 ±0.05	28.75 ±0.04	-
Y. enterocolitica NCTC 11175	0.63	14.06 ±0.05	11.43 ±0.13	16.15 ±0.17	21.26 ±0.15	22.52 ±0.04	21.22 ±0.06	-
B. subtilis RSKK 244	0.63	19.01 ±0.28	37.59 ±0.04	16.15 ±0.17	16.13 ±0.37	23.48 ±0.15	19.68 ±0.19	-
S.enteritidis RSKK 171	1.25	16.28 ±0.76	31.34 ±0.30	12.12 ±0.10	15.21 ±0.09	12.34 ±0.15	14.91 ±0.20	-
C. albicans ATCC 10231	2.50	12.84 ±0.20	-[d]	-	-	-	-	17.09 ±0.07
L. garvieae	1.25	13.48 ±1.14	33.17 ±0.06	15.11 ± 0.16	10.24 ± 0.01	32.81 ±0.05	23.05 ±0.14	-
V. alginolyticus	0.63	13.88 ±0.59	13.54 ±0.04	15.12 ±0.02	15.05 ±0.06	17.35 ±0.09	15.05 ±0.11	-
S. agalactiae Pas.Inst. 55118	1.25	13.02 ±0.55	37.34 ±0.30	18.85 ±0.26	15.75 ±0.18	32.67 ±0.16	9.89 ±0.21	-

[a]: MBC: Minimal Bactericidal Concentration
[b]: MFC: Minimal Fungicidal Concentration
[c]: Values are the mean of three separate experiments.
[d]: Indicates no antimicrobial activity.

Table 2. Absorbances at 700 nm of chitosan obtained from *C. quadrangula* ephippia

Concentration (µg/ml)	*C. quadrangula*
5000	0.039±0.01
2500	0.015±0.01
1250	0.014±0.01
625	0.006±0.01

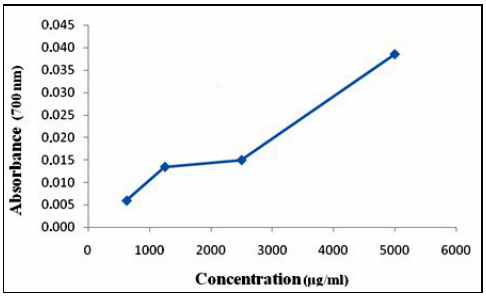

Figure 1. Reducing power activity of chitosan from *C. quadrangula*

3.2.2. DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity

In the test of the DPPH scavenging activity, a low IC_{50} value, which shows that half of the radicals were scavenged, expresses that the antioxidant has a high capacity. According to this, the IC_{50} values of chitosan from *C. quadrangula* ephippia and BHT were found to be 9.32±0.47 µg/ml and 0.04±0.01 µg/ml, respectively, in this study. The concentration-inhibition values of the chitosan from the ephippia of *C. quadrangula* are given in Table 3, and the DPPH scavenging activities are also given in Figure 2. When compared with the synthetic antioxidant, it was found that the chitosan had a lower activity. However, interest in natural antioxidants is increasing with each passing day because synthetic antioxidants are toxic and not natural [59]. From this aspect, chitosan, which is a natural product and has an antioxidant activity, can be preferred instead of synthetic antioxidants. Kuppusamy and Karuppaiah [60] reported that the DPPH scavenging activity of commercial chitosan was between 28.37% and 38.03% (at the concentration of 0.125-1 mg/ml), and it varied depending on the concentration, as in this study. Kaya et al. [31] revealed that chitosan and O-Carboxymethyl chitosan from *D. longispina* ephippia showed moderate free radical scavenging activity, with IC_{50} values of 23.01 and 56.43 mg/ml, respectively. The IC_{50} values of Colorado potato beetle chitosan was found to be 4.15 for adult and 10.38 for larvae [49]. Authors revealed that chitosan from adult potato beetles showed an inhibitory activity of 54.43% at the concentration of 5 mg/ml, and the chitosan from the larvae scavenged 33.05% of the DPPH radicals. It was recorded by Yen et al. [17] that free radical scavenging activity of fungal chitosan was 8.79-16.30 mg/ml and its scavenging ability ranged from 28.4 to 31.3%. at a concentration of 10 mg/ml. It is seen that chitosan from *C. quadrangula* ephippia exhibited a higher DPPH scavenging activity than larval potato beetle chitosan and fungal chitosan. Yen et al. [19] found that crab chitosan scavenged 28.4-52.3% of DPPH radicals at a concentration of 10 mg/ml. IC_{50} value was determined between 1.62 and 2.20 mg/ml for shrimp chitosan (*Metapenaeus monoceros*) at different concentrations (0-5 mg/ml) [56] and 10.68 mg/ml and 10.91 mg/ml, for the chitosans obtained from two cosmopolit Orthopteran species (*C. barbarus* and *O. decorus*) [50]. When comparing with these result, it is seen that *C. quadrangula* chitosan showed moderate DPPH radical scavenging activity by inhibiting 35.83% of the 1,1-diphenyl-2-picrylhydrazyl radicals at 5 mg/ml of concentration. Also, free radical scavenging activities of chitosans from different sources was similar to each other and this ability changed depending on source and concentration of chitosan.

Table 3. Concentration-inhibition values of chitosan obtained from *C. quadrangula* ephippia

	Concentration (µg/ml)	Inhibition (%)
Chitosan	312.5	14.13±0.85
	625	15.33±0.79
	1250	15.98±0.30
	2500	18.44±0.60
	5000	35.83±0.98

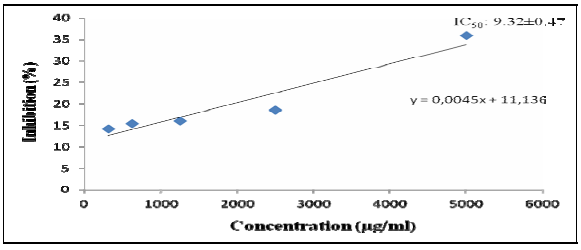

Figure 2. Scavenging activity of chitosan from *C. quadrangula* for DPPH

4. Conclusion

C. quadrangula ephippia chitosan was selected for antimicrobial and antioxidant activities for the following reasons; 1) it was fully characterized in earlier study [28], 2) antimicrobial and antioxidant studies were generally focused on shrimp, crab and crayfish chitosan but here antimicrobial and antioxidant activities of a new source was studied.

According to the results of this study, chitosan showed high antimicrobial activities against the human and fish pathogens tested. Moreover, chitosan showed better results against some pathogens than commercial antibiotics. In addition, chitosan exhibited a good DPPH scavenging activity and ferric ion reducing power. Chitosan obtained from the ephippia of *C. quadrangula* can be used as a new source to reduce or eliminate some problems resulting from the use of commercial antimicrobials and antioxidants.

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