

# In Vitro Antimicrobial and Antioxidant Activity of Chitosan Isolated from *Podophthalmus Vigil*

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## ABSTRACT

In the present study, Chitin has been extracted from *Podophthalmus vigil*. The obtained chitin was transformed into the more useful chitosan we evaluated the antimicrobial activity of different concentrations (25%, 50%, 75% and 100%) of chitosan against 10 species of bacteria and fungi by agar-disc diffusion method. As the concentration of chitosan increased, the antimicrobial effect was strengthened. In the conjugated diene method, the total antioxidant activity of chitosan was ranging from 18.37% to 79.58% at varying concentrations (0.1 to 10mg/ml). The reducing capability ranged from 0.12% to 0.48% at varying concentration (0.1 to 10mg/ml). The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging potential of chitosan ranged from 18.08% to 55.56% at varying concentrations (0.1 to 10mg/ml). The chelating ability of chitosan from *Podophthalmus vigil* on ferrous ions was ranging from 25.82% to 73.54% for the concentration between 0.1-10mg/ml. Hydroxyl radicals scavenging activity of chitosan (0.125- 2 mg/ml) was found to be higher than the ascorbic acid and the range was from 23.18% to 93.28%. Superoxide anion radical was ranging from 5.21% to 65.81% for the concentration between 0.125-2 mg/ml. overall, chitosan from *Podophthalmus vigil* was good in antimicrobial and antioxidant activity, and may be used as a source of antioxidants, as a possible food supplement or ingredient in the pharmaceutical industry.

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## INTRODUCTION

Nature has played an instrumental role in providing effective therapeutic entities. The historical relationship between mankind and the sea is usually for travelling, trading and as a nutritional source. Ocean cover more than 70% of the earth's surface but represents 95% of the biosphere (Ellis 2001). Chitin, found in the shell of crustaceans, the cuticles of insects, and the cell walls of fungi, is the second abundant biopolymer in the nature (Knorr 1984). Structurally, chitin is a straight-chain polymer composed of  $\beta$ -1, 4-N-acetylglucosamine and

classified into  $\alpha$ -,  $\beta$ - and  $\gamma$ -chitin (Cabib 1981; Cabib *et al.* 1988). Chitosan derived by partial N-deacetylation of chitin is also a straight-chain polymer of glucosamine and N-acetyl glucosamine (Muzzarelli *et al.* 1997).  $\alpha$ - Chitin, the most abundant in nature, has a structure of antiparallel chains and is found in the crab, shrimp and lobster whereas  $\beta$ -chitin found in squid has intrasheet hydrogen bonding by parallel chains (Jang *et al.* 2004; Minke and Blackwell 1978). However,  $\gamma$ -chitin found in the cell walls of fungi has a mixture of parallel and antiparallel chains, which is a combination of  $\alpha$ -chitin and  $\beta$ -chitin (Jang *et al.* 2004). Because chitin and chitosan possesses many beneficially biological properties such as antimicrobial activity (Kobayashi *et al.* 1990; Tokoro *et al.* 1989), biocompatibility, biodegradability, haemostatic activity and wound

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healing property, much attention has been paid to its biomedical applications (Farkas 1990; Fleet and Phaff 1981). Due to these unique properties, chitosan and its derivatives have been proposed for applications in biomedical, food, agriculture, biotechnology and pharmaceutical fields (Felse and Panda 1999; Kumar 2000; Shahidi *et al.* 1999). Currently, the natural compounds are the focus of biotechnological companies looking for new antimicrobial agents (Tripathi and Dubey 2004).

Chitosan is a kind of fine biomaterial. Antimicrobial properties of chitosan and chitosan derivatives have been widely explored (Shahidi *et al.* 1999; Lim and Hudson 2003; Rabea *et al.* 2003). Antioxidant properties of chitosan derivatives have been studied (Lin and Chou 2004; Xie *et al.* 2001; Xing *et al.* 2005). Furthermore, antioxidant properties of fungal chitosan from shiitake stipes have also been studied (Yen *et al.* 2007).

However, antioxidant properties of chitosan derived from crab shells are not available. Accordingly, the objective of this study was to assess the antimicrobial and antioxidant properties of chitosan prepared from *Podophthalmus vigil*.

## MATERIALS AND METHODS

### Collection of sample

Shell materials were obtained from the wastes of *Podophthalmus vigil* were collected from Mudasal odai landing centre, south east coast of India.

Then packed in plastic bags and stored at -20°C before and during transportation to the laboratory. Shells were scraped free of loose tissue from the crab wastes in laboratory, washed in double distilled water and dried at 60°C. Standard chitin and chitosan obtained from Himedia, Mumbai. All other reagents were of analytical grade.

### Preparation of chitosan

Chitin and chitosan were prepared from *Podophthalmus vigil* shell waste according to Takiguchi, 1991a and Takiguchi, 1991b.

### Isolation of chitin

#### Demineralization

20 gm of sample powder was demineralised with 300ml of 2N Hcl for 24 hours with constant stirring and thus filtered. The filtrate was again washed with distilled water and filtered till the liquid showed neutral PH. The filtrate was then dried in a vacuum dryer and weighed.

#### Deprotenization

The sample was then deprotenized with 300ml of 1N NaOH at 80°C for 24 hour with constant stirring. The NaOH was exchanged intermittently and the sample was washed with distilled water every time before adding fresh NaOH. After 24 hour the sample was filtered. The sample filtrate was washed as before and dried. The weight was noted.

### Isolation of chitosan by Takiguchi, (1991b)

The deprotenized sample was deacetylated with 250 ml of 40% NaOH, treated for 6 hours at 110° C constant stirring. The sample was filtered and the filtrate was washed as before, 200 ml of 10% acetic acid was added to the sample and stored for 12 hours at room temperature with constant stirring. The dissolving sample was reprecipitated by adding 40% NaOH to P<sup>H</sup> 10. The sample was then disalysed by deionised water. It was then centrifuged at 10,000 rpm for 10 minutes and the precipitate was freeze dried.

### Preparation of stock solution

Chitosan (1.0 mg) was dissolved in 2 ml of 1.0% (W/V) aqueous acetic acid solution. From this 0.25, 0.50, 0.75 and 1.0 ml was taken and made up to 1.0 ml by adding 1% acetic acid to prepare various concentrations containing 125µg, 250µg, 375µg and 500µg of chitosan sample corresponding to 25,50,75 and 100% respectively.

### Microbial strains used

Antibacterial effect of chitosan was determined against 6 different bacterial strains viz., *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia* and *Bacillus substilis*. Antifungal effect of chitosan was determined against 4 different fungal strains viz., *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans*, and *Penicillium citrinum*. These pathogenic strains were obtained from the department of Medical Microbiology (Raja Muthiah Medical College hospital) Annamalai University, Annamalai Nagar.

### Preparation of bacterial culture

Nutrient broth medium was prepared and sterilized in an autoclave at 15 lbs pressure. Nine bacterial species were incubated in the nutrient broth and incubated at 28 ± 2 °C for 24 hours. Nutrient agar medium was also prepared, autoclave and transformed aseptically in to sterile Petri dishes. On this, 24 hours old bacterial broth cultures were inoculated by using a sterile cotton swab.

### Antibacterial Assay

The antibacterial activity was studied using as test agent a range of 6 different strains of human pathogenic bacteria of which there were one antibiotic agent (tetracycline). *In vitro* antibacterial assay was carried out by disc diffusion technique (Bauer *et al.* 1966) in whatman No.1 filter paper discs with 4mm diameter were impregnated with known amount test samples. The disks were loaded each with 10 µl of the extract by first applying 5 µl with the pipette, allowed to evaporate, then applying another 5 µl, then drying again. The positive control contained of a standard antibiotic disc. Negative controls not comprised sterile disc only. The impregnated discs along with control (tetracycline) were kept at the center of agar plates, seeded with test bacterial cultures. The discs were then placed individually using a sterile forceps in

appropriate grids which were marked on the undersurface of the plated Petri plates and kept for incubation at room temperature (27°C±2) for 24 h. After incubation, plates were observed for zones of inhibition and recorded in millimeters.

#### Preparation of fungal culture

Stock cultures were maintained in Sabouraud Dextrose agar and 4 species of fungal pathogens was maintained in Sabouraud dextrose broth of 24 hours until used for antifungal activity.

61.36 g of sabouraud dextrose agar powder suspended in 1000 ml of distilled water and the pH was adjusted in 5.6 ± 0.2. The medium was boiled the dissolve completely and sterilized by autoclaving at 15 lbs at 121°C for 15minutes. 50g of Sabouraud dextrose broth powder suspended in 1000ml of distilled water and pH was adjusted in 5.6±0.2. The medium was boiled the dissolve completely and sterilized by autoclaving at 15lbs at 121°C for 15minutes.

#### Antifungal Assay

Stock cultures were maintained in sabouraud dextrose agar and 4 different species of fungal pathogen were maintained in Sabouraud Dextrose broth for 24 hours until used for antifungal activity. *In vitro* antifungal activity was determined by using the technique of (Bauer *et al.* 1966).

Six different species of fungal pathogen was inoculated by spread plate method using 0.1ml of 24hours old culture, maintained in Sabouraud Dextrose broth. Whatman No.1 filter paper (4mm) discs impregnated with test samples of the chitosan. The discs were loaded each with 10 µl of the extract by first applying 5 µl with the pipette, allowed to evaporate, then applying another 5 µl, then drying again. The positive control (streptomycin) contained a standard antifungal disc. The inhibition zone was measured after 32 hours at 30°C for the fungal plates. Antifungal activity was measured in term of diameter in term of diameter of zone (including the disc within) in mm.

#### Total antioxidant activity

The antioxidant activity was determined by conjugated diene method (Lingnert *et al.* 1979). Each chitosan sample (0.1-10mg/ml) in 0.2% acetic acid solution was mixed with 2ml of 10mM linoleic acid emulsion (P<sup>H</sup> 6.5) in test tubes and placed in darkness at 37°C to accelerate oxidation.

After incubation for 15hour, 6ml of 60% methanol in deionised water was added, and the absorbance of the mixture was measured at 234 nm against blank in a Hitachi U-2001 UV-VIS spectrophotometer.

The antioxidant activity was calculated as follows:

Antioxidant activity (%) =  $[\Delta A_{234} \text{ of control} - \Delta A_{234} \text{ of sample}] / \Delta A_{234} \text{ of control}] \times 100$ .

A control consisted of methanol and reagent solution without chitosan. An antioxidant activity value of 100% indicates the strongest antioxidant activity. Ascorbic acid, BHA and  $\alpha$ -tocopherol were used as a standard comparison.

#### Reducing power

Reducing power was determined according to the method of (Oyaizu 1986). Each chitosan sample (0.1-10mg/ml) was mixed with 2.5 ml of phosphate buffer (0.2M, P<sup>H</sup>-6.6) and 2.5 ml of potassium ferric cyanide (1%). Reaction mixture was kept in a water bath at 50°C for 20 minutes. After incubation, 2.5ml of trichloroacetic acid (10%) was added and centrifuged at 3,000 rpm for 10 minutes. From the upper layer, 2.5ml solution was mixed with 2.5ml solution was mixed with 2.5ml of distilled water at 0.5ml of ferric chloride (0.1%). Absorbance of all the solution was measured at 700nm. Increased absorbance indicated increased reducing power. Ascorbic acid and BHA were used for comparison.

#### Chelating ability on ferrous ions

Chelating ability was determined according to the method of (Dinis *et al.* 1994). Each chitosan sample (0.1-10mg/ml, 1ml) in 0.2% acetic acid solution was mixed with 3.7 ml of methanol and 0.1ml of 2mM ferrous chloride. The reaction was initiated by the addition of 0.2ml of 5mM ferrozine. Then, the mixture was shaken vigorously and left at room temperature for 10 minutes. Absorbance of the mixture was determined at 562nm against a blank. Citric acid and EDTA were used as standard. The inhibition percentage of ferrozine-Fe<sup>2+</sup> complex formation was calculated by using the formula given below:

$$\text{Metal chelating effect (\%)} = [(A_{\text{Control}} - A_{\text{Sample}}) / A_{\text{Control}}] \times 100$$

#### Scavenging ability on 1, 1-diphenyl 1-2-picryl hydroxyl radicals (DPPH)

The scavenging effect of chitosan on DPPH radical was examined using the modified method described earlier by (Shimada *et al.* 1992). Each chitosan sample (0.1-10mg/ml) in 0.2% acetic acid solution was mixed with 1ml of methanolic solution containing DPPH radicals, results/resulted in a final concentration of 10mM DPPH. The mixture was shaken vigorously and left to stand for 30min in the dark and the absorbance was then measured at 517nm against a blank. Ascorbic acid, BHA and  $\alpha$ -tocopherol were used as standard. The scavenging ability was calculated as follows:

Scavenging ability (%) =  $[\Delta A_{517} \text{ of control} - \Delta A_{517} \text{ of sample}] / \Delta A_{517} \text{ of control}] \times 100$ .

#### Hydroxyl radical assay

The rate of reaction of hydroxyl radical with a putative antioxidant was determined by the deoxyribose method (Halliwell *et al.* 1987). The reaction mixture containing chitosan (0.125, 0.25, 0.5, 1.0, 2.0 mg/ml) was incubated with deoxyribose (3.75 mM), H<sub>2</sub>O<sub>2</sub> (1 mM), FeCl<sub>3</sub> (100 IM), EDTA (100 IM) and ascorbic acid (100 IM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37°C. The reaction was terminated by adding 1 ml of TBA (1% w/v) and 1 ml of TCA (2% w/v) and then heating the tubes for 15 min in a boiling water bath. After cooling, the absorbance of the mixture was measured at 535 nm against reagent blank.

Ascorbic acid was used for comparison. The hydroxyl radical scavenging activity was calculated as following:

$$\text{Scavenging effect (\%)} = [(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}})] \times 100$$

Where  $A_{\text{blank}}$  presents the absorbance of the blank (distilled water, instead of the chitosan) and  $A_{\text{control}}$  presents the absorbance of the control (distilled water, instead of  $\text{H}_2\text{O}_2$ ). Each test replicated three times.

### Superoxide anion radical scavenging assay

The superoxide scavenging ability of chitosan was assessed by the method of Xing. The reaction mixture, containing chitosan (0.125, 0.25, 0.5, 1.0, 2.0 mg/ml), PMS (30  $\mu\text{M}$ ), NADH (338  $\mu\text{M}$ ) and NBT (72  $\mu\text{M}$ ) in phosphate buffer (0.1 M, pH 7.4), was incubated at room temperature for 5 min and the absorbance was read at 560 nm (Shimadzu UV-Vis Spectrophotometers) against a blank. Ascorbic acid was used for comparison. Each test replicated three times. The capability of scavenging to superoxide radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = (1 - A_{\text{sample 560nm}} / A_{\text{control 560nm}}) \times 100.$$

## RESULTS

### Antimicrobial activity

Antibacterial and antifungal activities of chitosan from *Podophthalmus vigil* screened against 6 human pathogenic bacterial strains and 5 fungal pathogens are presented in Table 1 & 2.

Effect of chitosan on pathogenic bacteria revealed that, the highest activity (13.04 mm) was observed against *Escherichia coli* with highest (100%) concentration. Regarding at 25, 50, 75% concentration maximum activity (12.04, 11.09, 11.06 mm) was found against *Escherichia coli*. The lowest activity was found with 25% concentration against *Bacillus subtilis*. Chitosan does not show inhibition against *Pseudomonas aeruginosa* at all concentrations. At 25, 50, 75% concentrations of chitosan does not show inhibition against *Staphylococcus aureus*.

For fungi, the highest activity (20.87 mm) was observed against *Penicillium citrinum* with highest (100%) concentrations. The lowest activity was found with 75% concentration against *Aspergillus flavus*. Chitosan does not show inhibition against *Aspergillus fumigatus* at all concentrations. At 25, 50% concentrations of chitosan does not show inhibition against *Staphylococcus aureus*.

### Total antioxidant activity

In the conjugated diene method, the total antioxidant activity of chitosan was ranging from 18.37% to 79.58% at varying concentrations (0.1 to 10mg/ml). It showed consistent antioxidant activity with increased concentration whereas ascorbic acid and BHA reported 79.58% and 86.41%

of antioxidant activity respectively at the highest concentration (Fig.1). Ascorbic acid and BHA were used as standard.

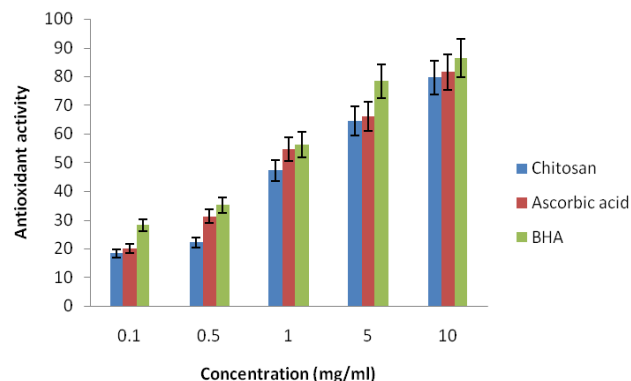


Fig. 1: Scavenging effects of the chitosan from *Podophthalmus vigil*, ascorbic acid and BHA on total antioxidant activity. Each test replicated three times. The bar represented  $\pm$  SD.

### Reducing power

The reducing capability of chitosan from *Podophthalmus vigil* was assessed based on the measurement of  $\text{Fe}^{3+}$ - $\text{Fe}^{2+}$  transformation. At 0.1mg/ml of chitosan showed slight reducing power of 0.12%. The reducing power of chitosan was correlating well with increasing concentration. But values remained lower than, those for ascorbic acid and BHA 0.38% and 0.91% at 0.1mg/ml respectively (Fig. 2). The reducing capability ranged from of 0.12% to 0.48% at varying concentration (0.1 to 10mg/ml). Ascorbic acid and BHA were used as standard.

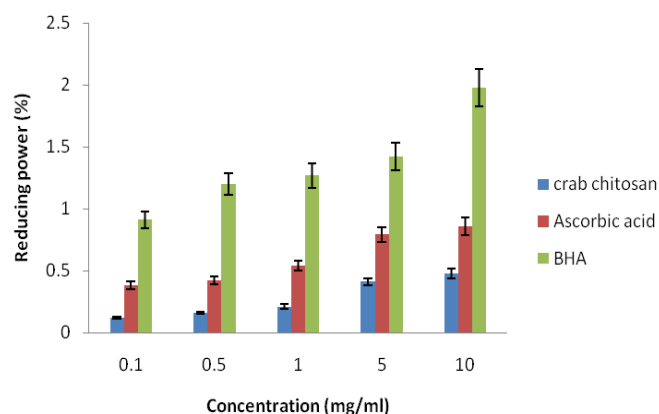


Fig. 2: Reducing power of the chitosan from *Podophthalmus vigil*, ascorbic acid and BHA. Each test replicated three times. The bar represented  $\pm$  SD.

### Scavenging ability on DPPH radicals

The scavenging ability of chitosan from *Podophthalmus vigil* on DPPH radicals was reported at 55.56% at 10mg/ml. Ascorbic acid and BHA showed moderate to high scavenging abilities of 60.34% and 85.40% respectively. Thus the antioxidant activity of chitosan from *Podophthalmus vigil* was found to be a moderate scavenger for DPPH radicals. The DPPH radical scavenging potential of chitosan ranged from 18.08% to 55.56% at varying concentrations (0.1 to 10mg/ml). Ascorbic acid and BHA were used on standard (Fig. 3).

**Table. 1:** Antibacterial Activity Of Chitosan.

Sl.No	Microorganisms	Concentration of chitosan				Positive control	Negative control
		500 µg (100%)	375 µg (75%)	250 µg (50%)	125 µg (25%)		
1.	<i>Staphylococcus aureus</i>	8.17±1.21	-	-	-	20.07±1.21	-
2.	<i>Salmonella typhi</i>	9.02±1.08	8.10±1.19	8.24±0.27	8.07±1.15	17.03±0.84	-
3.	<i>Escherichia coli</i>	13.04±1.11	12.04±0.35	11.09±0.43	11.06±0.32	18.04±0.12	-
4.	<i>Klebsiella pneumoniae</i>	10.07±0.06	9.07±1.16	8.07±1.20	8.11±0.64	18.15±1.08	-
5.	<i>Pseudomonas auergunosa</i>	-	-	-	-	16.09±0.53	-
6.	<i>Bacillus substilis</i>	9.17±1.17	8.12±0.36	8.18±0.28	8.01±0.86	13.07±1.17	-

(-) = No zone of inhibition

Results indicates zone of inhibition in mm indicate the distance from the border of the disc to the edge of the clear zone.

Values are given as mean ± SD of three experiments.

Positive control (Tetracycline100 µg); Negative Control (Sterile Disc)

**Table. 2:** Antifungal Activity of Chitosan.

Sl.No	Microorganisms	Concentration of chitosan				Positive control	Negative control
		500 µg (100%)	375 µg (75%)	250 µg (50%)	125 µg (25%)		
1.	<i>Aspergillus flavus</i>	4.43±0.87	2.38±0.65	-	-	16.17±0.53	-
2.	<i>Candida albicans</i>	10.64±0.29	9.27±0.44	8.39±0.47	8.77±1.12	12.08±0.73	-
3.	<i>Aspergillus fumigatus</i>	-	-	-	-	11.27±0.49	-
4.	<i>Penicillium citrinum</i>	20.87±0.59	18.17±0.84	12.56±1.25	10.07±0.67	15.47±1.18	-

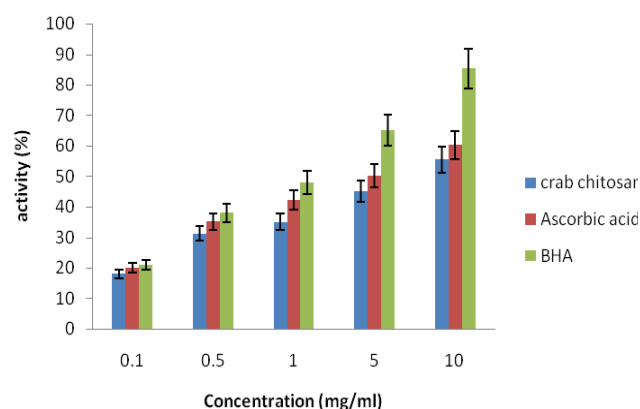
(-) = No zone of inhibition

Results indicates zone of inhibition in mm indicate the distance from the border of the disc to the edge of the clear zone.

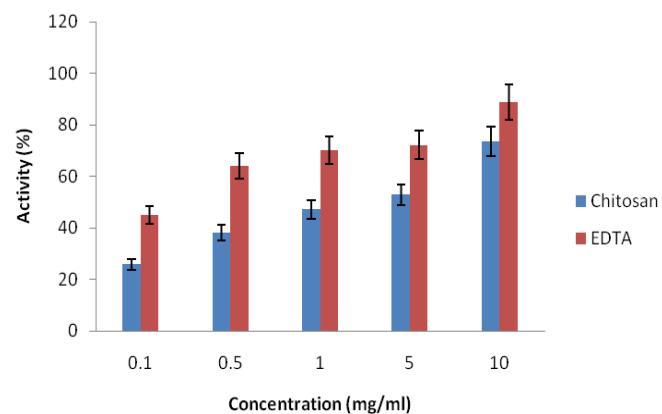
Values are given as mean ± SD of three experiments.

Positive control (Streptomycin-100 µg)

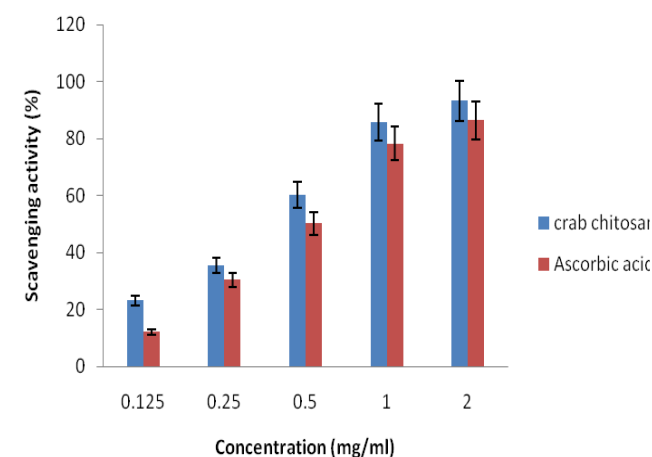
Negative Control (Sterile Disc)



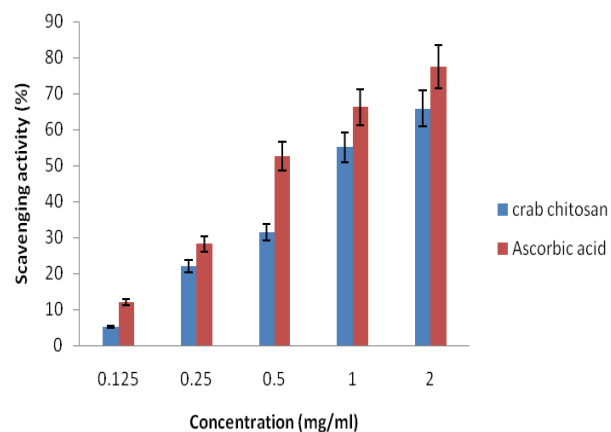
**Fig. 3:** Scavenging activities of the chitosan from *Podophthalmus vigil*, ascorbic acid and BHA on DPPH radical. Each test replicated three times. The bar represented ± SD.



**Fig. 4:** Ferrous ions chelating ability of the chitosan from *Podophthalmus vigil* and EDTA. Each test replicated three times. The bar represented ± SD.



**Fig. 5:** Scavenging effects of the chitosan from *Podophthalmus vigil* and ascorbic acid on hydroxyl radicals. Each test replicated three times. The bar represented ±SD.



**Fig. 6:** Scavenging effects of the chitosan from *Podophthalmus vigil* and ascorbic acid on Superoxide anion radicals. Each test replicated three times. The bar represented ±SD

### Chelating effect on ferrous ions

The chelating ability of chitosan from *Podophthalmus vigil* on ferrous ions was ranging from 25.82% to 73.54% for the concentration between 0.1-10mg/ml (Fig. 4). However, the chelating effect of EDTA (0.1-10mg/ml) was found to be higher than the chitosan and the range was from 45.12% to 88.75%. EDTA was used as standard.

### Hydroxyl radical scavenging activity

The chitosan from the shell of *Podophthalmus vigil* had obvious scavenging activity and exhibited a stronger concentration-dependent inhibition of deoxyribose oxidation. Moreover, it had stronger Scavenging activity on hydroxyl radical than ascorbic acid (Fig. 5). Scavenging effect of chitosan was 60.25% at concentration of 0.5 mg/ml, whereas scavenging effect of ascorbic acid was 50.15% at same concentration. Hydroxyl radicals scavenging activity of chitosan (0.125- 2 mg/ml) was found to be higher than the ascorbic acid and the range was from 23.18% to 93.28%. Ascorbic acid was used as standard.

### Superoxide anion radical scavenging activity

The inhibitory effect of the chitosan from the shell of *Podophthalmus vigil* on superoxide anion radical was ranging from 5.21% to 65.81% for the concentration between 0.125-2 mg/ml (Fig. 6). However, the scavenging effect of Ascorbic acid (0.125- 2 mg/ml) was found to be higher than the chitosan and the range was from 12.12% to 77.41%. Ascorbic acid was used as standard.

## DISCUSSION

As a kind of macromolecule polymer, Chitosan seems to be unable to pass the outer membrane of bacteria, since this membrane functions as an efficient outer permeability barrier against macromolecules. Therefore, direct access to the intracellular parts of the cells by Chitosan is unlikely. The antibacterial mechanisms of Chitosan suggested being, the positive charge of the amino group at C-2 resulted in a polycationic structure which can be expected to interact with the predominantly anionic components (lipopolysaccharides, proteins) of the microorganisms surface (Helander *et al.* 2002). The interaction resulted in great alteration of the structure of outer membrane (Helander *et al.* 1998), which caused release of major proportion of proteinaceous material from the cells (Vaara and Vaara 1983). As a result, the chitosan was easier to interact with anionic components of cell surface, and exhibited higher inhibitory activities. According to literature (Jeon *et al.* 2001; Ueno *et al.* 1997), chitosan possesses antimicrobial activity against a number of Gram-negative and Gram-positive bacteria. This study has been conducted to assess inhibitory effects of chitosan. The effectiveness of chitosan bacteriostatic properties were tested against bacterial strains.

Yen *et al.* (2008) reported that crab chitosan showed moderate to high antioxidant activities of 58.3-70.2% at 1mg/ml

and high antioxidant activities of 79.9-85.2% at 10mg/ml. In addition, the antioxidant activities of crab chitosan C60, C90 and C120 correlated with their N-deacetylation times. In the present investigation, chitosan showed consistent antioxidant activity with increased concentration. Antioxidant activity of chitosan from *Podophthalmus vigil* was 18.37-79.58% at 0.1 to 10mg/ml, shown lesser than that of both the fungal and crab chitosan which may due to the differences in N-deacetylation times.

One important mechanism of antioxidant involves the scavenging of hydrogen radicals. DPPH has a hydrogen free radical and shows a characteristic absorption at 517nm (Brand-Williams *et al.* 1995). After encountering the proton-radical scavengers, the purple color of the DPPH solution fades rapidly (Yamaguchi *et al.* 1998). In this study, DPPH was used to determine the proton-scavenging activity of the various disaccharide chitosan derivatives.

Yen *et al.* (2008) reported that fungal chitosan scavenged DPPH radicals by 28.4-53.5% at 10mg/ml, obviously chitosan from crab shells and *shiitake stipes* was also not an effective scavenger for DPPH radicals. Yen *et al.* (2007) reported the scavenging ability of crab chitosan C60 on DPPH radicals was 28.4% at 10mg/ml, whereas these of other crab chitosan were in the range of 46.4-52.3%. The scavenging ability of chitosan from *Podophthalmus vigil* was 55.56% at 10mg/ml. However, at 10mg/ml, BHA and Ascorbic acid showed scavenging abilities of 85.40% and 60.34% respectively.

The reducing power of different molecular weights of  $\gamma$ -ray treated chitosan has determined by potassium ferricyanide reduction method showed that low molecular weight  $\gamma$ -ray treated chitosan exhibited high reducing power and the reducing power increased with the increases of chitosan concentration (Feng *et al.* 2008). In the present study, the reducing power of chitosan from *Podophthalmus vigil* was 0.48 at 10mg/ml. However, BHA and ascorbic acid showed reducing power of 1.98 and 0.86 at 10mg/ml respectively. It shows that chitosan from *Podophthalmus vigil* was not effective in reducing power. It seems that reducing powers of chitosan also correlated with their N-deacetylation times (Yen *et al.* 2007).

The  $\text{Fe}^{2+}$  chelating ability of chitosan mainly comes from the presence of amino groups, which contain lone pairs that help to form chitosan- $\text{Fe}_2^+$  complexes (Guzman *et al.* 2003). And  $\text{Fe}_2^+$  chelating ability of low molecular weight chitosan was more pronounced than high molecular weight chitosan (Xing *et al.* 2005b).

Huang *et al.* (2005) added EDTA to the reaction system to release the  $\text{Fe}^{2+}$  that had become chelated by the chitosan, and the scavenging activity against hydroxyl radical decreased. EDTA is a more effective chelate for  $\text{Fe}^{2+}$  than chitosan, and so released  $\text{Fe}^{2+}$  can react with  $\text{H}_2\text{O}_2$  to generate excessive amounts of hydroxyl radicals, which result in the decrease of radical scavenging activity. Therefore,  $\text{Fe}^{2+}$  chelating directly correlate to the scavenging behavior of chitosan.

In the present investigation, the chelating ability of chitosan from *Podophthalmus vigil* on ferrous ion was 73.54% at

10mg/ml and 25.82 % at 0.1mg/ml respectively. The chelating abilities of chitosan correlated with their N-deacetylation times. However, EDTA showed high chelating ability of 88.75% at 10mg/ml. Yen *et al.* (2007) reported that 1mg/ml chelating abilities of fungal chitosan were all chelated. Lin and Chou (2004) reported that the chelating abilities of N-alkylated disaccharide chitosan derivative were less than that observed with EDTA.

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. It is an initial free radical formed from mitochondrial electron transport system (Blokina *et al.* 2003). Superoxide anion radicals are produced by a number of cellular reactions, including various enzyme systems, such as lipoxigenases, peroxidase, NADPH oxidase and xanthine oxidase. They play an important role in the formation of other cell damaging free radicals, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems. In the present study a superoxide radical scavenging assay was based on the capacity of chitosan to inhibit the reduction of nitro blue tetrazolium (NBT). Significant scavenging of superoxide radical was evident at all the tested concentration of chitosan. At 0.5mg/ml, the scavenging percentage of chitosan from *Podophthalmus vigil* against superoxide radical was 31.47%.

Although superoxide is a relatively weak oxidant, it decomposes to form stronger reactive oxidative species, such as single oxygen and hydroxyl radicals, which initiate peroxidation of lipids (Dahl and Richardson 1978). In the present study, chitosan effectively scavenged superoxide in a concentration dependent manner. Further, superoxides are also known to indirectly initiate lipid peroxidation as a result of H<sub>2</sub>O<sub>2</sub>. Hydroxyl radical scavenging activity of chitosan was obtained in the deoxyribose system. In this system, chitosan exhibited a concentration-dependent inhibition of deoxyribose oxidation. Earlier, numerous workers (Halliwell *et al.* 1987) have employed this system to assess the biological activity of various natural plant-derived biomolecules. Smith *et al.* (1992) reported that molecules that can inhibit deoxyribose degradation are those that can chelate iron ions and render them inactive or poorly active in a Fenton reaction. In the present study, in another assay system, we found chitosan had moderate chelating effect. So, it is likely that the chelating effect of chitosan on metal ions may not be responsible for the inhibition of deoxyribose oxidation.

## CONCLUSION

Based on this study, Chitosan show significant antimicrobial and antioxidant activity. The antimicrobial activity of chitosan in different concentration was studied. The effect of chitosan against human pathogens can be successfully determined by the agar disc diffusion assay. According to our results, as the concentration of chitosan increased, the antimicrobial effect was strengthened. Antioxidative properties of the various chitin and chitosan extracts are of great interest in food industry, since their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidants by natural ones. Owing to its excellent protective features exhibited in antioxidant activity tests,

the chitin and chitosan extracts from the crustacean species could be concluded as a natural source that can be freely used in the food industry. This study identifies opportunities to develop value added products from crustacean processing by-products with different biological activity such as antioxidant, antibacterial and antifungal properties.

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