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Chapter 3

Antimicrobial properties of crustacean chitosan on *Staphylococcus aureus* (ATCC 6538) and *Candida albicans* (ATCC 10231)

Pulin Kerketta*
Assistant Professor, Department of Zoology, Gossner College, Ranchi, Jharkhand, India
*Corresponding author email: kerkettapulin7@gmail.com
DOI: https://10.5281/zenodo.8330980
https://orcid.org/0009-0007-2551-4322

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ABSTRACT:- In recent years, there has been a rise in drug resistance in various microbes. This led to the necessity of the discovery of new bioactive compounds with antibacterial and antifungal activity. Thus, an attempt has been made to extract chitosan from shells of freshwater crabs, *Maydelliathelphusa masoniana,* and freshwater prawn, *Macrobrachium dayanum*. These crustaceans are commonly found in Jharkhand's ponds, rivers, and paddy fields. These are edible crustaceans, relished by the local people of Jharkhand. An assay of chitosan was done at different concentrations (1.25 mg/ml, 2.5 mg/ml, 3.75 mg/ml, and 5 mg/ml) using the agar well diffusion method against *S. aureus* (ATCC 6538) and *C. albicans* (ATCC 10231). The result showed the antibacterial activity against *S. aureus* was a zone of inhibition formed by *M. masoniana* chitosan, was 12.0±0.2 mm at 1.25 mg/ml, 13.03±0.05 mm at 2.5 mg/ml,14. 16±0.15 mm at 3.75 mg/ml and 15.13±0.15 mm at 5 mg/ml. The positive control, Ciprofloxacin (5µg/disc) showed 25.03±0.05 mm inhibition zone. *M. dayanum* chitosan against *S. aureus* showed 14.03±0.20 mm at 1.25 mg/ml, 15.16±0.11 mm at 2.5 mg/ml,16.16±0.15 mm at 3.75 mg/ml and 17.20±0.20 mm at 5 mg/ml inhibition zone. The positive control, Ciprofloxacin (5µg/disc) showed a 28.03±0.05 mm inhibition zone.

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Antifungal activity against *C. albicans* revealed that the zone of inhibition formed by *M. masoniana* chitosan was 12.33±0.58 mm at 5 mg/ml and no inhibition zone was observed at 3.75 mg/ml, 2.5 mg/ml, 1.25 mg/ml chitosan concentration and positive control, Streptomycin (10 µg disc^{-1}) against the fungus. The zone of inhibition formed by *M. dayanum* chitosan was 14.33±0.58 mm at 5 mg/ml. No inhibition zone was observed at 3.75 mg/ml, 2.5 mg/ml, and 1.25 mg/ml chitosan concentration, and positive control, Streptomycin (10-µg disc^{-1}) against the fungus. Comparison between the inhibition zone of chitosan (5 mg/ml) from *M. masoniana* and *M. dayanum* showed significantly higher efficacy of chitosan extracted from *M. dayanum* against *S. aureus* (ATCC 6538) ($p<0.001$) and *C. albicans (*ATCC 10231) ($p<0.05$) than chitosan extracted from *M. masoniana.*

Keywords: *M. masoniana*, *M. dayanum*, Chitosan, antimicrobial properties

INTRODUCTION

Antimicrobial agents are becoming vital day by day. There are many microorganisms that are directly or indirectly associated with human infections and diseases. Some common bacterial diseases are gastrointestinal illness or diarrhea caused by eating foods contaminated. The food that is not kept hot enough (60ºC, 140ºF or above) or cold enough (7.2ºC, 45ºF or below) may carry enterotoxins produced by some strains of *S. aureus*[1]. Ingestion of such food causes human intoxication, which has symptoms of nausea, vomiting, abdominal cramping, and prostration[1]. One of the common fungal diseases is candidiasis caused by *C. albicans*. It colonizes the oropharyngeal cavity, gastrointestinal and vaginal tract, and healthy individual skin[2]. In the case of its overgrowth, it caused symptoms like digestive issues, fatigue, and joint pain. *C. albicans* can cause infections that range from superficial infections of the skin to life-threatening systemic infections[3]. Antimicrobial materials are being developed to prevent harmful bacteria and fungi from spreading. These synthetic antimicrobial materials can effectively control the growth and reproduction of hazardous bacteria but at the same time can have side effects on their administration. In recent years, studies about antimicrobial materials have been focused on natural materials such as chitosan. Chitosan is a derivative of chitin,

the most abundantly found biopolymer polysaccharide found in crustacean shells. Chitosan is a copolymer comprised of glucosamine and N-acetyl-glucosamine linked through â 1-4 glycosidic bonds and obtained by deacetylation of chitin. (Fig.1)

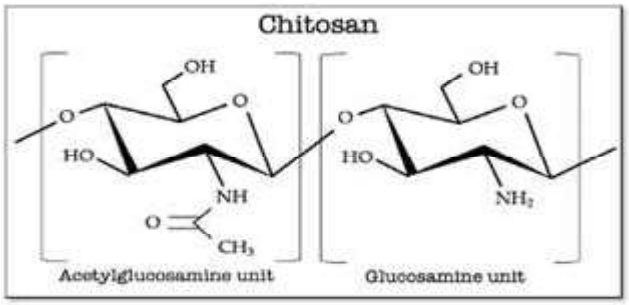

Fig. 1: Molecular structure of chitosan

Crustaceans like crabs, *Maydelliathelphusa masoniana,* and prawns, *Macrobrachium dayanum* are found in freshwater bodies, like rivers, ponds, and paddy fields of Jharkhand. Local people use them as food and as medicines to cure various diseases. The traditional belief of therapeutic drinks prepared by baking the shell of a crab which enriched the calcium supply of the body was reported by Sriphuthorn (2000)[4]. Chinlampianger *et al.* (2013)[5] reported that the Adi tribe from Arunachal Pradesh gives *Penaeus indicus* in old age and diabetic people. For curing several diseases like colds, coughs, genito-urinary disorders, renal disorders, and body weakness,[6] prawns like *Penaeus monodon* are being used.

The objective of the research:

The present research investigation was made in search of biological properties by antimicrobial efficacy of chitosan at different concentrations, extracted from shell waste of freshwater crab, *Maydelliathelphusa masoniana* and freshwater prawn, *Macrobrachium dayanum* against *S. aureus* (ATCC 6538) and *C. albicans* (ATCC 10231).

MATERIALS AND METHODS:

Preparation of Chitosan from crustacean shells:

The crabs and prawns were collected from the local market in Jharkhand. The shells were washed thoroughly with water then the shells were dried and crushed using a mortar and pestle. Chitosan was extracted by demineralization, deproteinization, and deacetylation[7, 8].

Antimicrobial Assay:

Bacteria, *S. aureus* (ATCC 6538) and fungus, *C. albican*s (ATCC 10231) were collected from the Department of Microbiology, Yugantar Bharti Analytical and Environmental Engineering Laboratory, Sidroul, Namkom, Ranchi, Jharkhand. Antimicrobial activity was tested using Ciprofloxacin 5µg/disc and Streptomycin 10 µg/disc as a positive control for bacteria and fungus respectively.

Nutrient broth medium was prepared and sterilized in an autoclave at 15 lbs pressure. Microbial species were incubated in the nutrient broth and incubated at 34°C for 24 hours. Nutrient agar medium was also prepared, autoclaved, and transferred aseptically into sterile Petri dishes. During these, 24 hours bacterial broth cultures were inoculated by using a sterile cotton swab. The antibacterial activity of the individual bacterial strains was tested using the Agar Well Diffusion method [9]. Well of 6 mm diameter were made aseptically in the plates. 24 hours old microbial broth cultures were inoculated using a sterile cotton swab on sterile nutrient agar plates. Using a micropipette, a solution of different concentrations (5 mg/ml, 3.75 mg/ml, 2.5 mg/ml, 1.25 mg/ml) of chitosan and 0.2 % acetic acid as negative control was loaded in the respective wells. Ciprofloxacin disc (5µg) for *S. aureus* and Streptomycin (10 µg) disc for *C. albicans* were placed using sterile forceps, as a positive control. The plates were incubated at 34°C for 24 hours in an upright position. The antimicrobial assay was carried out in triplicate. After incubation at 34°C for 24 hours, the zone of inhibition was measured in millimeters. The results obtained were analyzed using the student's t-test.

RESULT

Antimicrobial activity

Table 1: Antibacterial activity of chitosan against *S. aureus* (ATCC 6538)

Sl. No.	Chitosan Concentration (mg/ml)	Zone of inhibition(mm)	
		M.masoniana chitosan	*M.dayanum* chitosan
1	1.25	12.0±0.2	14.03±0.20
2	2.5	13.03±0.05	15.16±0.11
3	3.75	14.16±0.15	16.16±0.15
4	5.00	15.13±0.15	17.2±0.20***
5	Positive Control (Ciprofloxacin 5µg/disc)	25.03±0.05	28.03±0.05
6	Negative Control (0.2 %Acetic acid)	-	-

(-) = No zone of inhibition, Values are given as mean ±SD of three experiments.
*** $p<0.001$ or significant at 0.1%

Fig. 2 & 3: Antibacterial activity of chitosan from *M. masoniana and M. dayanum* at different concentrations of (1.25 mg/ml, 2.5 mg/ml, 3.75 mg/ml, and 5 mg/ml) with positive control, ciprofloxacin (5 µg/ disc) and negative control (0.2 % acetic acid) in *Staphylococcus aureus* (ATCC 6538).

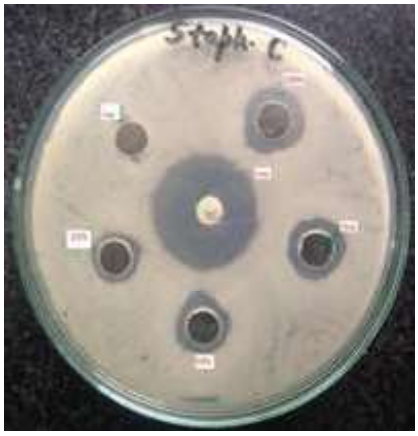

Fig. 2 : Effect of *M.masoniana* chitosan on *S.aureus*

Fig. 3: Effect of *M.dayanum* chitosan on *S.aureus*

Table 1 represents the effect of chitosan from *M. masonaina* and M. *dayanum* on *S. aureus* (ATCC 6538). In the case of *M. masoniana* chitosan, a maximum zone of 15.13±0.15 mm was formed in 5 mg/ml chitosan concentration whereas, in the concentration of 3.75 mg/ml, 2.5 mg/ml and 1.25 mg/ml against the same bacteria the zone of inhibition was found 14.16±0.15 mm, 13.03±0.05 mm, and 12.0±0.20 mm respectively (Fig.2). For positive control, antibiotic Ciprofloxacin (5µg $disc^{-1}$) showed 25.03±0.05 mm diameter clear zone of inhibition against *S. aureus* (ATCC 6538). In the case of *M. dayanum* chitosan, a maximum zone of 17.20±0.20 mm was formed in 5 mg/ml chitosan concentration whereas, in the concentration of 3.75 mg/ml, 2.5 mg/ml and 1.25 mg/ml against the same bacteria the zone of inhibition was found 16.16±0.15 mm, 15.16±0.11mm, and 14.03±0.20 mm respectively(Fig.3). For positive control, antibiotic Ciprofloxacin (5µg $disc^{-1}$) showed 28.03±0.05 mm zone of inhibition against *S. aureus* (ATCC 6538). For negative control, 0.2% acetic acid solution was used which showed no zone of inhibition.

Table 2: Antifungal activity of chitosan against *C. albicans* (ATCC 10231)

Sl. No.	Chitosan Concentration (mg/ml)	Zone of inhibition(mm)	
		M. masoniana chitosan	*M. dayanum* chitosan
1	1.25	-	-
2	2.5	-	-
3	3.75	-	-
4	5.00	12.33±0.58	14.33±0.58*
5	Positive Control (Streptomycin 10 µg/disc)	-	-
6	Negative Control (0.2 %Acetic acid)	-	-

(-) No zone of inhibition, Values are given as mean±SD of three experiments.
*p<0.05 or significant at 5%

Fig. 4 & 5: Antifungal activity of chitosan at different concentrations of (1.25 mg/ml, 2.5 mg/ml, 3.75 mg/ml, and 5 mg/ml) extracted from *M. masoniana* and *M. dayanum* with positive control, streptomycin (10 µg/disc) and negative control (0.2 % acetic acid) from in *Candida albicans* (ATCC 10231).

Fig. 4: Effect of *M. masoniana* chitosan on *C. albicans*

Fig. 5: Effect of *M. dayanum* chitosan on *C. albicans*

Table 2 represents the effect of chitosan from *M. masonaina* and *M. dayanum* on the pathogenic fungus, *C. albicans* (ATCC 10231). It revealed that maximum inhibition zone of 12.33±0.58 mm and 14.33±0.58 mm was formed by *M. masonaina* and *M. dayanum* respectively in the concentration of chitosan solution of 5mg/ml, whereas in 3.75 mg/ml, 2.5 mg/ml and 1.25 mg/ml chitosan concentration against the same fungus no zone of inhibition was observed. For positive control, Streptomycin (10 µg $disc^{-1}$) was used which showed no zone of inhibition

against *Candida albicans* (ATCC 10231). For negative control, 0.2% acetic acid solution was used which showed no zone of inhibition. (Fig.4 &5). On comparing the zone of inhibition formed, chitosan concentration at 5 mg/ml extracted from *M. dayanum* had significantly higher efficacy than chitosan extracted from *M. masoniana* against *Candida albicans* (ATCC 10231) ($p<0.05$).

Comparing between inhibition zone of chitosan (5 mg/ml) from *M. masoniana* and *M.dayanum* showed higher efficacy of chitosan extracted from *M. dayanum* against *Staphylococcus aureus* (ATCC 6538) ($p<0.001$) and *Candida albicans* (ATCC 10231) ($p<0.05$) than chitosan extracted from *M. masoniana.*

DISCUSSION

The present results align with the findings of Prabhu & Natarajan (2012)[10] where chitosan exhibited concentration-dependent antimicrobial activity. Islam *et al.* (2011)[11] reported that the antibacterial activity of chitosan from crab shells from Khulna, Bangladesh against *S. aureus* showed 13 mm, 13 mm, 12 mm, and 10 mm zones of inhibition at chitosan concentrations of 1000 µg/mL, 800 µg/mL, 600 µg/mL and 400 µg/mL respectively. The above result corroborated with the study of the present observation where the similar zone of inhibition 12.0±0.2 mm in *M. masoniana* was reported at 1.25 mg chitosan concentration against *S. aureus* (ATCC 6538). The similarity in antibacterial efficacy may be due to similarity in extraction process of both the chitosans by chemical method of demineralization, deproteinization, and deacetylation. According to Kamala *et al.* (2013)[12,] the antibacterial activity of crude chitosan from *Parapeneopsis stylifera* shrimp shell which was deproteinized at 60ºC against, *S. aureus* showed 6.4 mm and 8.9 mm zone of inhibition at 500 µg/mL and 1mg/mL chitosan concentration respectively. The reason for the higher antibacterial efficacy of chitosan extracted from *M.dayanum* may be due to performing the deproteinization process at a higher temperature of 80ºC, resulting in the production of chitosan with a higher degree of deacetylation having better antimicrobial efficacy. Prabhu & Natarajan, (2012)[10] reported that antifungal activity of *Podophthalmus vigil* chitosan against fungal strains *C. albicans,* showed 10.64±0.29 mm, 9.27±0.44 mm, 8.39±0.47 mm and 8.77±1.12 mm inhibition zone at chitosan concentration of 500 µg/ml, 375 µg/ml, 250 µg/ml, and 125 µg/ml

respectively. In comparison with the present study, chitosan of freshwater prawns, *M. dayanum* showed higher antifungal efficacy with a larger inhibition zone of 14.33±0.58 mm at 5 mg/ml chitosan concentration than the chitosan extracted from Litopenaeus *vannamei* which showed inhibition zone of 4 mm against *Candida albicans.* Affes, S. *et al.*[13] demonstrated that high molecular weight shrimp chitosan, especially C120, possesses higher antibacterial and antifungal potentials. Saravanan, A. *et al.*[14] reported significant antibacterial activity of shrimp shell chitosan, against *V. cholera* by producing an inhibition zone of 18 mm. Amor *et al.*[15] reported that chitosan extracted from different insects showed good antibacterial activity against *S. aureus*.

CONCLUSION

The chitosan exhibited considerable antimicrobial activities against pathogenic microorganisms *Staphylococcus aureus (*ATCC 6538) and *Candida albicans* (ATCC 10231). The inhibition zone varied with the concentration. The mechanism of antimicrobial activity of chitosan is attributed to three ways[16]:

1. The electrostatic interaction between positively charged protonated amino NH^{3+} groups of chitosan and negatively charged cell membrane leads to disruption of the cell membrane /cell wall of the microbe.
2. Interaction of chitosan with microbial DNA, inhibiting mRNA and protein synthesis.
3. Chitosan, being a chelating agent, blocks the flow of nutrients and causes cell death.

The current investigation is allied with the observations of several researchers [10-12].

The application of chitosan can thus be used as an antimicrobial agent against a greater range of microbes, opening a wide range of possibilities.

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