INHIBITORY PROPERTY OF CHITOSAN EXTRACT FROM SHRIMP AGAINST BACTERIA ISOLATED FROM DIABETIC FOOT ULCER

Abstract

Treatment of diabetic foot ulcers (DFUs) is considerably challenging. Impaired wound healing may be caused by poor vascularization and dysfunction of the extracellular matrix, which leads to poor re-epithelialization and increased risk of infection. In this study, we evaluated the inhibitory potential of shrimp-derived chitosan against bacteria isolated from DFU wounds. Vannamei shrimp (*Litopenaeusvannamei*) presents potential as a raw material of micro chitosan production, because its shell contains chitin and chitosan. Chitosan is a natural polysaccharide with specific properties, including non-toxicity, biodegradability, and biocompatibility. It is obtained from the process of chitin deacetylation and has a free amino group (NH2) that makes this polycationic polymer a suitable antibacterial agent. The aim of this study was to extract chitosan from shrimp and to evaluate its antibacterial properties against organisms isolated from diabetic foot ulcer wounds. Shrimp was collected and chitosan extraction was extracted. Isolation of bacteria was done from swabs of diabetic ulcer wounds. Analysis of antibacterial activity of shrimp-derived chitosan was done by well diffusion method. It was found that chitosan has potent anti-microbial properties against pathogens associated with diabetic ulcers. DFUs are a serious complication of diabetes that can lead to amputation if not treated properly. In addition to its antibacterial properties, chitosan is also non-toxic, biodegradable, and biocompatible. This makes it a promising candidate for use in wound dressings and other medical applications. Overall, the study showed that chitosan obtained from vannamei shrimp has the potential to be developed into a new and effective treatment for DFUs.

INTRODUCTION

Diabetic foot ulcers (DFUs) represent one of the most debilitating complications of diabetes mellitus, affecting approximately 6.8% of diabetic patients globally. Diabetes is a long-term condition characterized by high blood glucose levels and abnormal protein and fat metabolism, as a result of the insufficient pancreatic secretion of insulin or under-utilization of insulin produced. There are two types of diabetic conditions, type 1 is a condition in which the pancreas does not produce sufficient insulin; type 2 is a condition in which the body's cells are resistant to the effects of insulin that is produced, and as a result, insulin production gradually declines over a period of time. Diabetes that is not under control can cause damages in numerous organs resulting in comorbidities such as heart attacks, strokes, loss of function

in the kidneys, and damage to tiny and major blood vessels as well as nerves, which may result in amputations of the lower limbs.

Chitin is the second most abundant biopolymer on earth with its deacetylated derivatives, chitosan has several applications in biomedical and pharmaceutical fields. Chitin is found in the exoskeletons of insects, the cell walls of fungi, and certain hard structures in invertebrates and fish.

Chitosan is a sugar that comes from the outer skeleton of shellfish, including crab, lobster, and shrimp. It's used as medicine and in drug manufacturing. Chitosan is a fibrous substance that might reduce how much fat and cholesterol the body absorbs from foods. It also helps blood clot when applied to wounds. People use chitosan for high blood pressure, high cholesterol, obesity, wound healing, and many other purposes, but there is no good scientific evidence to support many of these uses.

Diseases and disease agents that were once thought to be controlled by antibiotics are returning in new leagues resistant to these therapies. Resistance to multiple drugs was first detected among enteric bacteria—namely, *Escherichia coli*, *Shigella* and *Salmonella*—in the late 1950s to early 1960s. Such strains posed severe clinical problems and cost lives, particularly in developing countries.

The extensive use of antimicrobials and close contact among sick patients creates a fertile environment for the spread of antimicrobial-resistant germs. Selection of resistant microorganisms is exacerbated by inappropriate use of antimicrobials.

Antimicrobial Activity of Chitosan

Chitosan's antibacterial activity depends on bacterial species, pH, molecular weight, concentration, and solubility. It inhibits both Gram-positive (e.g., *Staphylococcus aureus*) and Gram-negative (e.g., *Pseudomonas aeruginosa*) bacteria by disrupting cell membranes via electrostatic interactions between its positively charged polycations and negatively charged bacterial membranes. Chitosan also disrupts bacterial biofilms, enhancing its effectiveness against chronic wound infections. Modifications like increased deacetylation or creating micro-chitosan (micro-polymer) with stronger ions improve its antibacterial properties

Its antimicrobial properties have gained significant attention for inhibiting various microorganisms. However, chitosan’s antibacterial activity is limited to acidic environments

because it is poorly soluble at high pH levels. This activity is often observed when chitosan is dissolved in acidic media like acetic, lactic, or hydrochloric acid.

Diabetic foot ulcers have a high recurrence rate, with about 40% of patients developing new ulcers within a year of healing. Randomized controlled trials (RCTs) targeting this high-risk group could be small yet yield significant benefits for individuals and healthcare systems.

The aim of the study was to isolate chitosan from shrimp and to test its antibacterial potency against microorganisms isolated from diabetic foot ulcer.

MATERIALS AND METHODS:

I. COLLECTION OF SHRIMPS:

Fresh shrimp was collected from the local market in Neyyattinkara, Thiruvananthapuram and the exoskeleton (shells) about 10g was extracted from the shrimp.

II. EXTRACTION OF CHITOSAN FROM THE SHRIMP:

Washed, air-dried shrimp shells were demineralized with 1.5 N HCl at 100°C for 1 hour, washed until neutral pH. Prawn shells were deproteinized with 0.5% NaOH at 100°C for 30 minutes, then 3% NaOH at 100°C for 30 minutes, washed until neutral, and decolorized to form chitin cake. Chitosan was made by deacetylating chitin with 42% NaOH at 95°C for 1.5 hours, washed until pH < 7.5, air-dried, and confirmed by dissolving in 1% acetic acid.

III. COLLECTION OF DIABETIC FOOT ULCER (SWAB) SAMPLE

The diabetic foot ulcer swabs (DFU Swab) were collected aseptically from patients visiting the Department of Podiatry, NIMS Hospital, Neyyattinkara, Thiruvananthapuram. The Samples were labelled properly and were stored at 4℃ until further experiments.

IV. ISOLATION OF BACTERIAL COLONIES

Isolation of microorganisms was done by serial dilution method. Collected samples were suspended into PBS (3ml), dilution from 10^{-1} to 10^{-3} were prepared using sterilised PBS (pH 7.4). 0.1ml of each sample was spread on a nutrient agar plate. These plates were then incubated at 37℃ for 24 hours.

V. PURE COLONIES ISOLATION

After incubation, distinct colonies were isolated and purified by repeated streaking on agar plates, then labelled. Pure colonies were stored in nutrient agar slants at 4°C.

VI.GRAM STAINING OF THE ISOLATED BACTERIA: Transfer a drop of culture to a slide, spread into a thin 1.5cm film, and heat-fix over a burner. Stain with crystal violet for 1 minute, rinse, then apply iodine for 1 minute and rinse. Decolorize with ethanol, rinse, and stain with safranin for 30 seconds. Rinse, air-dry, and examine under a 100x oil immersion microscope. Gram-positive bacteria appear deep violet; Gram-negative appear pink/red.

VII. ANALYSIS OF ANTIBACTERIAL ACTIVITY OF SHRIMP-DERIVED CHITOSAN BY WELL DIFFUSION METHOD

Petri plates with 20ml Muller Hinton Agar were seeded with bacteria. Five 10mm wells were bored, filled with 10%, 20%, and 30% chitosan, and incubated at 37C for 24 hours. Antibacterial activity was measured by the inhibition zone diameter. Amoxicillin (50µl) served as positive control, acetic acid as solvent control, and zones were measured after overnight incubation at 37°C.

RESULTS

Chitosan powder was soluble immediately in 1 % acetic acid (v/v) with a ratio of 1 g /100 ml. This result indicated that the sample was chitosan with a degree of deacetylation above 50 %

1. ISOLATION OF BACTERIA FROM THE SWABS

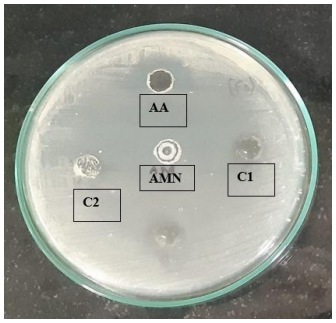
Isolation of microorganisms was done by serial dilution method. Collected samples were suspended into PBS (3ml), dilution from 10^{-1} to 10^{-3} were prepared using sterilised PBS (pH 7.4).

After incubation, morphologically distinct colonies were found, and the selected colonies were isolated and purified by repeated streaking in the agar plates. The isolated colonies were the labelled as D1, D2, D3, D4 and D5. The pure colonies were maintained in nutrient agar slants

Table 1: Antibacterial activity of chitosan against bacteria isolated from DFU wounds

III. ANALYSIS OF ANTIBACTERIAL ACTIVITY OF CHITOSAN

Petri plates containing Muller Hinton Agar medium were seeded with bacterial isolates of D1-D5. Wells of approximately 5mm were bored using a well cutter and different concentration of chitosan (250 and 500 µg/mL) were added into the wells. Amoxicillin was added as positive control and acetic acid was added as negative control. And the plates were then incubated at 37℃ for 24 hours. After 24 hours of incubation the anti-



bacterial activity was assessed in all plates by observing and measuring the zone of inhibition (mm) (Table 1) (Fig. 1- 5). The results showed that chitosan derived from shrimp has potent antibacterial properties against isolates D1, D2 and D3. However, chitosan was ineffective against D4 and D5 strains (Table 1) (Fig. 1-5).

Isolated strain	**Diameter of Inhibitory zone (mm)**			
	CHITOSAN		**Negative control (acetic acid)**	**Positive control (Amoxicillin)**
	250 µg/ml	**500 µg/ml**		
D1	13	16	15	25
D2	12	13	10	15
D3	11	13	11	30
D4	-	-	13	26
D5	_	_	_	33

Fig 1 Analysis of antibacterial activity of chitosan against D1 colony. AMN-Amoxycillin, AA-Acetic acid, C1 250µg/ml and C2-500µg/ml

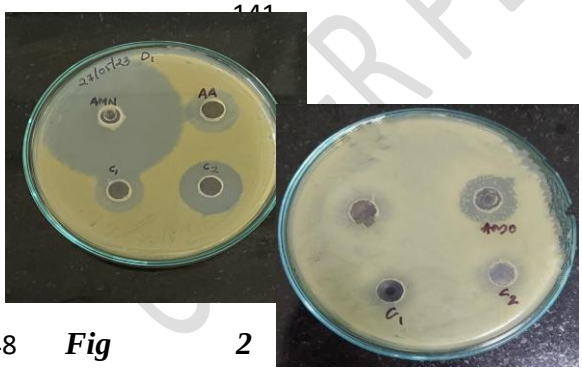


Fig 2 Analysis of activity of chitosan against D2 colony. AMN- Amoxycillin, AA-Acetic acid, C1 250µg/ml and C2-500µg/ml

Fig.3 Analysis of antibacterial activity of chitosan against D3 colony. AMN- Amoxycillin, AA-Acetic acid, C1 250µg/ml and C2-500µg/ml

Fig 4 Analysis of antibacterial activity of chitosan against D4 colony. AMN- Amoxycillin, AA-Acetic acid, C1 250µg/ml and C2-500µg/ml

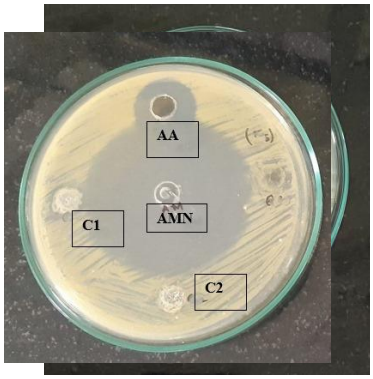


Fig 5 Analysis of antibacterial activity of chitosan against D5 colony. AMN- Amoxycillin, AA-Acetic acid, C1 250µg/ml and C2-500µg/ml

DISCUSSION

Diabetic foot ulcer (DFU) is one of the major complications associated with diabetic mellitus. Neuropathy and ischaemia are two major etiological factors leading to DFU. Intensive care should be taken for patients with DFU for the prevention of amputation. These ulcers tend to heal slowly, and healing can be complicated by polymicrobial infection and heavy exudate formation which place these patients at a higher risk for limb amputation

Clinical studies have shown that wound with a high bacterial burden are less likely to heal. Therefore, it is assumed that if the microbial load is minimized, the wounds will heal faster avoiding many of the complications of DFU. Also, studies have shown that approximately 30% of DFU will be colonized with MRSA. DFU infected with drug resistant bacteria lead to worse outcomes which increases the rate of amputation and mortality.

Foot ulcers affect about 15% of patients with diabetes, remain one of the most common causes of hospitalization and comprise 85% of lower-limb amputations in diabetic patients. The risk of leg amputation is correlated with the grade and stage of the wound and other determinants such as peripheral sensomotoric neuropathy, ulceration, infection and peripheral vascular disease.2 The management of diabetic foot ulcers (DFU) requires a multi-disciplinary approach, including control of the diabetes, orthotic shoes, off-loading devices, wound care and surgery in selected cases.3 However, treatment of DFU remains challenging because of frequent unsatisfactory results from both surgical and non-surgical treatments.

Isosorbide dinitrate (ISDN) is an organic nitrate and a vasodilator with effects on arteries and veins. The chemical name of ISDN is 1,4:3,6-dianhydro-2,5-di-O-nitro-D-glucitol. The principal pharmacological action of ISDN is relaxation of the vascular smooth muscle and consequent dilatation of peripheral arteries and veins. This process is mediated by the synthesis of nitric oxide (NO). Chitosan composed of randomly distributed Beta-D-glucosaminyl-(1->4)-beta-D-glucosamine and N-Acetyl-D-Glucosamine is a polymer with good haemostatic properties, natural biocompatibility, low cytotoxicity, antimicrobial activity and excellent biodegradable properties.4 The objective of this study was to evaluate whether the combination of a vasodilation agent and a barrier therapy such as 10% chitosan gel topically applied takes advantage of each agent's individual characteristics to render it suitable for management of DFU.

Although the combination of ISDN and chitosan, or ISDN as monotherapy presented statistically significant differences compared to placebo, they only showed a 'small effect' (Cohen's d test) compared to conventional treatment using microcyn and sterile gauze to clean the wound. Although progression of the ulcer was the primary outcome in the present study, we consider that complete closure of the ulcer should be more important to avoid infection and the consequent recurrence. Our results showed that the combination of ISDN and chitosan increased the number of patients who reach complete ulcer closure compared with placebo, but the sample was small to make definitive statements. Histological analysis showed an increase in vWF, desmin, VEFG-A and α-SMA in accordance with the healing evolution and not by the type of intervention. The increase in VEFG-A, secondary to wound healing, might suggest an increase in angiogenesis, which allows the activity of growth factors and cytokines.

Biomaterials are being extensively used in regenerative medicine including tissue engineering applications, as these enhance tissue development, repair, and help in the process of angiogenesis. Wound healing is a crucial biological process of regeneration of ruptured tissue after getting injury to the skin and other soft tissue in humans and animals. Besides, the accumulation of microbial biofilms around the wound surface can increase the risk and physically obstruct the wound healing activity and may even lead to amputation. Hence, in both acute and chronic wounds, prominent biomaterials are required for wound healing along with antimicrobial agents. This review comprehensively addresses the antimicrobial and wound healing effects of chitosan, chitin, cellulose acetate, hyaluronic acid, pullulan, bacterial cellulose, fibrin, alginate, etc. based wound dressing biomaterials fabricated with natural resources such as honey, plant bioactive compounds, and marine-based polymers. Due to their excellent biocompatibility and biodegradability, bioactive compounds derived from honey, plants, and marine resources are commonly used in biomedical and tissue engineering applications. Different types of polymer-based biomaterials including hydrogel, film, scaffold, nanofiber, and sponge dressings fabricated with bioactive agents including honey, curcumin, tannin, quercetin, andrographolide, gelatin, carrageenan, etc., can exhibit significant wound healing process in, diabetic wounds, diabetic ulcers, and burns, and help in cartilage repair along with good biocompatibility and antimicrobial effects. Among the reviewed biomaterials, carbohydrate polymers such as chitosan-based biomaterials are prominent and widely used for wound healing applications followed by hyaluronic acid and alginate-based biomaterials loaded with honey, plant, and marine compounds. This review first provides an overview of the vast natural resources used to formulate different biomaterials for the treatment of antimicrobial, acute, and chronic wound healing processes.

Short-term exposure of DFU bacterial isolates to AL-AgNPs showed significant growth inhibition, biofilm disruption, ROS accumulation, membrane leakage, and DNA gyrase toxicity. Surface phytochemicals of AL-AgNPs interacted with DNA gyrase B's ciprofloxacin-binding site, confirmed by in silico studies. TEM analysis revealed varied antibacterial mechanisms against multidrug-resistant Gram-positive and Gram-negative bacteria, suggesting AL-AgNPs as a potent antimicrobial for diabetic wound infections. Further in vivo wound healing studies are needed.

Modern DFU wound dressings should maintain moisture, prevent infection, and promote healing with minimal scarring. Chitosan, a broad-spectrum antimicrobial biopolymer, targets microbes via multiple mechanisms, reducing resistance compared to single-target antibiotics.

Composed of glucosamine and N-acetyl glucosamine, chitosan is biocompatible, biodegradable, and haemostatic. Combined with antibacterial nanoparticles and hydrophilic HA, it supports moist wound healing and scarless recovery. This study aimed to evaluate chitosan's antibacterial properties against DFU bacteria for optimized wound dressings

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