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Evaluation of Antibacterial and Antifungal Activity of Chitosan in Integument of Cockroaches

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Abstract

Introduction: Due to drug resistance and toxic side effects observed following administration of many antibacterial drugs, novel strategies are highly needed for treatment of bacterial diseases. Chitosan, as an important immune system stimulator, is found in cockroaches that are living in contaminated sites. The aim of this study was to extract the chitosan from nymph and adult stage of both *Blattella germanica* and *Periplaneta americana* and to evaluate its antibacterial and antifungal activities.

Methods: The mature form of *B. germanica* and the nymph and adult forms of *P. americana* were killed by CO2 gas, and washed and dried at 60ºC. Then, they were mechanically ground in a mixer and passed through 20 mesh size. Finally, chitosan was extracted from shrimp processing discards. Chitosan was dissolved in lactic acid 1% and its effects against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Vibrio cholerae,* methicillin-resistant *S. aureus*, *Candida albicans*, and *Aspergillus* were evaluated.

Results: Chitosan extracted from cockroaches showed antibacterial effects on gram-positive bacteria especially *S. aureus* and *E. coli*. However, it had no impact on fungi.

Conclusion: The findings of the current study revealed that chitosan had excellent antibacterial activities.

Keywords: Chitosan, Antifungal, Antibacterial, Insects

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Introduction

Since a vast majority of bacterial species developed resistance against antibacterial drugs which are highly toxic, novel strategies are required to be employed in this respect. The annual cost of drug-resistant infections is estimated to be $20 billion in surplus health care costs and 8 million extra hospital days in the United States.[1] Because the majority of insects such as cockroaches live in contaminated areas, they appear to have developed effective strategies to deal with pathogens.[2] Chitosan as a natural polysaccharide has been widely used in pharmaceutical excipients.[3] This material is produced by the deacetylation of chitin. Chitin is a natural polymer composed of randomly dispersed β-(1-4)-linked D-glucosamine. This biopolymer is a major component of not only organisms like crustaceans, fungi, and insects,[4] but also of invertebrates' integument.[5] Chitin can also be produced by chemical and biological methods.[6,7] Chitin annual turnover was estimated to range from 10^{10} to 10^{11} tones.[8,9] Additionally, it can be readily obtained by simple extraction.[6] In addition, it contains proteins, minerals, lipids, and pigments.[10] Chitosan has characteristic features such as bioadhesive nature and hydrophilic properties. Besides, it can be used as potent carrier for brain-targeted drugs, transdermal films and wound healing biodegradable grafts, as well as antimicrobial and stabilizing constituent of liposomes. Due to these properties,

chitosan, as one of the most promising renewable polymeric materials for a variety of applications, has received greater attention than before. According to some studies, the unique capabilities of chitosan have made it a strong pharmaceutical option.[11-13] Notably, it facilitates the transportation of polar drugs through the epithelial surfaces and is regarded as both biocompatible and biodegradable.[14]

Recently, various studies have focused on the strong antimicrobial effects of chitosan against different groups of microorganisms, from bacteria[15] to fungi,[16] parasites,[17-20] and yeasts.[21] Therefore, investigations on the chitosan and its antimicrobial potential have recently become of particular interest.[22-26] Despite numerous studies on the antimicrobial effects of chitosan, researchers have not yet come up with a consensus result in this respect. Therefore, further studies should be conducted accordingly.

The major commercial sources of chitin and chitosan come from wastes of marine food production and also crab and shrimp shells.[27-30] Moreover, this material can be produced from exoskeleton of arthropods as well as the cell walls of fungi and yeasts.[5] Insects like *Periplaneta americana* and *Blattella germanica* are the potential sources of chitosan due to their high reproduction rate.[31] Because of the low content of minerals and total protein in insects' body, less acidic and alkaline materials are required to extract the chitin and chitosan.[32] According to what has previously been mentioned, this study aimed at extracting chitin and chitosan from nymph and adult cuticle of cockroaches (*B. germanica* & *P. americana*) and assessing the anti-bacterial and anti-fungal properties of the extracted chitosan.

Materials and Methods

Preparation of Insect Corpses

In this study, the nymph and adult forms of *B. germanica* and *P. americana* were obtained from the Laboratory of Medical Entomology, Tehran University of Medical Sciences. Adult and nymph forms were killed by CO2 gas. Subsequently, insect corpses were washed by distilled water (DW) and dried at 60°C. Then, they were mechanically crushed in a mixer and passed through 20 mesh size.

Demineralization

Chitosan was extracted from shrimp processing discards using a method described by Chang et al[27] Demineralization step was accomplished by mixing 5 g of *B. germanica* and *P. americana* integument dry powder with 1M HCl at 100°C for 30 minutes followed by rinsing with DW until natural pH was obtained. This demineralized insect sample was subsequently washed (with 99% ethanol) and air dried at room temperature.

Deproteinization

This step was performed using alkaline treatment. The demineralized integuments dry powder was deproteinized with 1M NaOH solution at 80°C. This step was repeated several times during 24 hours. The chitin product was filtered through 20 µM mesh and washed with DW until the pH became neutral.

Deacetylation

In order to deacetylate the chitin, NaOH 50% was used at 100°C for 4 hours. Then, the alkali was drained off and washed thoroughly with DW until the pH was less than 7.5 and subsequently dried at ambient temperature (30±2°C).

Antimicrobial Susceptibility Test

The suspensions of bacteria were prepared in sterile phosphate-buffered saline (PBS) and standardized to 1×10^6 CFU/mL. The antimicrobial activities of the chitosan solution were tested against *Staphylococcus aureus* (ATCC; 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Vibrio cholerae* (ATCC; 39315), and methicillin-resistant *S. aureus* (ATCC: 33591) by disk diffusion method (Bauer et al, 1996). Bacterial suspension was initially prepared and inoculated onto the entire surface of a Mueller-Hinton agar (MHA) (diameter, 90 mm) with pH of 6.9 using a sterile cotton-tipped swab to form an even lawn.

The plate was air-dried for 15 minutes and five sterile paper disks (6 mm in diameter; BD, Becton Dickinson Diagnostic Systems) impregnated with 20 µL diluted chitosan solution (0.4% & 0.8% for chitosan concentration, respectively) were loaded on the surface of each MHA plate using a sterile pair of forceps. Then, the plates were incubated aerobically at 37°C for 24 hours. The diameter of inhibition zone was measured after 24 hours of incubation in the usual manner.

Disc Diffusion Method for Fungi

Disk diffusion method was performed for fungi against *Candida albicans* and *Aspergillus* species on yeast nitrogen base glucose detection (YNBG). Five disks papers, impregnated with chitosan solution, were placed over the MHA medium.

Data Analysis

All steps of this work were carried out in triplicate. Data were analyzed using SPSS (statistical package for the social sciences) software, version 18. In addition, ANOVA and independent student *t* test were used.

Results

Demineralization

Five grams of the yielded powder of integument from *B. germanica* and *P. americana* were treated with 1M HCl in order to extract the chitin (Table 1). The average of the remaining material were 2.12 and 2.17 mg after demineralization step for nymph and adult forms of *B.*

Table 1. The Integument Composition of *Blattella germanica* and *Periplaneta americana*

Species Names	Stage	Chitosan Composition (%)	Chitin Composition (%)	Mineral Composition (%)	Protein Composition (%)
B. germanica	Nymph	5	5.6	42.4	52
	Adult	5.2	6.2	43.4	44
P. americana	Nymph	3.6	4.4	42.6	53
	Adult	11	14.8	46.6	38.6

germanica while being 2.13 and 2.33 mg for nymph and adult forms of *P. americana*.

Deproteinization

The average amounts of the remaining chitin after deproteinization from *B. germanica* in nymph and adult forms were 0.28 and 0.31 mg whereas in *P. americana*, nymph adult forms, they were 0.22 and 0.74 mg, respectively.

Deacetylation

Following deacetylation of *B. germanica* and *P. americana*, to extract the chitosan in NaOH 50 %, the average amounts of the remaining chitosan were 0.25 and 0.26 mg in nymph and adult forms of *B. germanica* and also 0.18 and 0.55 mg in nymph and adult forms of *P. americana*, respectively.

Antibacterial Test

In this test, both gram-positive and gram-negative bacteria were tested. The chitosan showed different degrees of antibacterial activity against *S. aureus, E. coli*, and *P. aeruginosa*. However, no significant antibacterial activity was observed against *V. cholerae* and methicillin-resistant *S. aureus*. Table 2 shows inhibition zone for different concentrations of chitosan against the tested bacteria. The chitosan did not show any activity against *C. albicans* and *Aspergillus* species.

Discussion

Chitin is a major part of the insect's cuticle, which always covalently binds to minerals and proteins. The most

Table 2. Inhibition Zone Diameter (mm) Following Administration of Different Concentrations of Chitosan for Selected Bacteria

	Species	Developmental Stage	Diameter Inhibition Zone (mm) 500 µg/mL	Diameter Inhibition Zone (mm) 1 mg/mL
S. aureus	*B. germanica*	Nymph	<0.5*	12
		Adult	<0.5*	11.5
	P. Americana	Nymph	<0.5*	11.2
		Adult	<0.5*	11
	Chitosan ** (Standard)		<0.5*	10.5
	Control (lactic acid 1%)		<0.5*	10.6
Pseudomonas species	*B. germanica*	Nymph	<0.5*	11.2
		Adult	<0.5*	10.4
	P. Americana	Nymph	<0.5*	9.6
		Adult	<0.5*	8.8
	Chitosan (standard)		10.5	11.2
	Control (lactic acid 1%)		<0.5*	<0.5*
E. coli	*B. germanica*	Nymph	<0.5*	10.4
		Adult	<0.5*	9.6
	P. americana	Nymph	<0.5*	<0.5*
		Adult	<0.5*	9.3
	Chitosan (standard)		<0.5*	10
	Control (lactic acid 1%)		<0.5*	<0.5*
V. cholera	*B. germanica*	Nymph	<0.5*	<0.5*
		Adult	<0.5*	<0.5*
	P. Americana	Nymph	<0.5*	<0.5*
		Adult	<0.5*	<0.5*
	Chitosan (standard)		<0.5*	<0.5*
	Control (lactic acid 1%)		<0.5*	<0.5*
Methicillin- resistant *S. aureus*	*B. germanica*	Nymph	<0.5*	<0.5*
		Adult	<0.5*	<0.5*
	P. americana	Nymph	<0.5*	<0.5*
		Adult	<0.5*	<0.5*
	Chitosan (standard)		<0.5*	<0.5*
	Control (lactic acid 1%)		<0.5*	<0.5*

frequently used method for chitin extraction from insects has two steps: 1) an acidic step, to eliminate minerals (also named demineralization step); and 2) a basic step, to eliminate the proteins from cuticle (also referred to as deproteinization step).[4]

In this study, the amount of chitosan extracted from adults and nymphs of the two spices was completely different. Compared to the results obtained in previous studies on crab and shrimp shells,[27] based on the percentage of the obtained compounds, these results suggest that the methods used for chitosan production from crab and shrimp shells was also an effective method for chitosan extraction from adult and nymph forms of *P. americana* and *B. germanica* as well.

There are various studies focused on the antimicrobial activity of different chitosans and their derivatives from different sources. The results of all these studies proved that chitosan inhibited the development of a wide variety of bacteria.[25,33-36] In fact, different concentrations of both types of chitosan used in the present study demonstrated antimicrobial activity on the selected bacteria.[37] However, chitosan could not inhibit the development of methicillin-resistant *S. aureus* species. Applying chitosan in cooperation with Ag + nanoparticles has rendered antibacterial activities.[38] In fact, the use of nanoscale compounds because of the availability to the drug target, could increase its effect. There are some examples in this regard.[20,39]

Preliminary studies demonstrated that chitosan extracted from *P. americana* was more effective than what extracted from *B. germanica*[40] while in the current experiment, the chitosan extracted from *B. germanica* showed better level of efficacy. Furthermore, chitosan was shown to have better effects on gram-positive than on gram-negative bacteria.

In general, there has been little research on antifungal activity of chitosan. Kendra et al assessed the antifungal effects of different acetylation grade of chitosan against *Fusarium solani* in an *in vitro* study. The results indicated that chitosan had maximum antifungal activity especially the high-molecular-weight chitosan.[41] In the current study, no inhibitory activity of chitosan was observed in fungi. Chitosan was found to have a greater antifungal activity at pH = 4.0.[42] However, in order to be passed through the anti-bacterial filter, chitosan solution was prepared at pH = 7.0. It is worth to mention that this solution is extremely dense at pH = 4.0 and will not pass through the anti-bacterial filter.

Conclusion

According to the results of the recent study, chitosan had antimicrobial effects. For this reason, it is expected that further studies to be carried out on this compound to determine its antibacterial or antimicrobial effects on other bacteria and microorganisms. Additional studies should also be undertaken to determine the toxic effects of this combination in order to eventually introduce it as an antimicrobial agent.

Ethical Approval

Considering that human and animal samples were not used in this study, there was no need for moral confirmation.

Competing Interests

The authors declare that there is not any conflict of interests.

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