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Determination of the MIC and MBC of Chitosan from Cockroach and Cricket Against Some Bacterial Isolates

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Abstract: To determine the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of chitosan from cockroach and cricket against bacterial isolates, *Staphylococccus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, Streptococcus pyogenes, Bacillus subtilis* and *Pseudomonas aeruginosa.* The antimicrobial efficacy of chitosan was determined by the standard method of Clinical and Laboratory Standards Institute (CLSI). Different concentrations were prepared and the MICs were calculated by tube dilution method in triplicates. The MBCs were determined by the lowest concentration that kills 99.9% of the initial bacterial population. The data generated were analyzed by ANOVA and Turkeys post hoc test using spss soft ware. The MIC of cockroach chitosan against *S. aureus, B. subtilis, K. pneumoniae, S. pyogenes, P. aeruginosa* and *S. epidermidis,* were found to be 240, 220, 0, 260, 240 and 0mg/ml while the MIC of cricket chitosan against the organisms were found to be 240,240,240,0, 220 and 280mg/ml. The MBC of chitosan from cockroach against the organisms were found to be 260, 260, 0, 280, 260 and 0 mg/ml while that of cricket were found to be 240, 240, 280, 0, 240 and 300 mg/ml. This study revealed that chitosan from *P. Americana* and *A. domestica* can be used as disinfectants in wounds, since they act against *S. aureus, B. subtilis, K. pneumoniae, S. pyogenes, P. aeruginosa* and *S. epidermidis* which are organisms commonly found in wound.

Keywords: Chitosan, Antimicrobial Activity, Bacterial Isolates, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC)

1. Introduction

Since the global population is constantly growing, people are exploring more renewable resources because they posess extraordinary huge biomass and they are promising resources of proten, lipid and chitin [19]. In addition, some species of insects provide special substrates, like antimicrobial peptides, which make them more valuable [13]. The American cockroach (*Periplanata americana*) is one of the world's most thriving insect in the tropics, subtropic and temperate regions. In china, *P. americana* has been used to deal with ulcers, wounds, edema hypochondriac pain and palpitations of thousands of years [15]. Previous researches have proven that *P. americana* possess several outstanding biological properties, including antibacterial ability, antioxidant, inflammatory and cancer [20, 25]. For now, various substances including protein, fatty acid, antimicrobial peptide, chitin and chitosan, have been separated from *P. americana* so that they could be studied separately [11].

Edible insects such as cricket has gained recent attention as emerging protein sources to help alleviate the demand of food in a growing world population [16]. In addition to the consumption and production and development of cricket based products, technology has been developed for enzymatic hydrolysis of cricket protein [8] which provides concentrated protein powders rich in essential amino acids that can be incorporated into low or poor quality protein foods to enhance the overall protein content and quality. Most importantly, a large by product of this manufacturing technology is the chitin rich exoskeleton of crickets. The giant cricket (*Braclytrupes portentosus*) and the African field

cricket (*Gryllus bimaculatus*) have been used successfully as sources of chitin and chitosan [5].

Chitin is the second most abundant natural polysaccharide after cellulose. Chitin consist of N-acetylglucosamine and D-glucosamine units that are linked by β (1,4) glucosidic bonds [14]. Chitin exists mainly as a structural component in arthropods, some mollusks, and cell walls of fungi and algae [3].

Chitosan is the deacetylated derivative of chitin. During the deacetylation, N-acetyl-D-glucosamine units would be changed to D-glucosamine units. This is because large number of amino groups distributing along the polysaccharide chains, chitosan possesses several excellent properties, such as biocompatibility, non-toxicity, biodegradability and the ability of anticancer [7] therefore, chitosan has been applied in various fields like agriculture, waste water treatment, food industry, cosmetic and biopharmaceutics [28]. The development of *P. americana* chitosan could add value to this "pest" helping to change people's attitude to it.

Wound is a type of injury which happens relatively quickly in which the skin is torn, cut, or punctured (an *open* wound), or where blunt force trauma causes a contusion (a *closed* wound). In pathology, it specifically refers to a sharp injury which damages the epidermis of the skin. There are many types of wounds that can damage the skin including abrasions, lacerations, rupture injuries, punctures, and penetrating wounds. Many wounds are superficial requiring local first aid including cleansing and dressing. Some wounds are deeper and need medical attention to prevent infection and loss of function, due to damage to underlying structures like bone, muscle, tendon, arteries and nerves. The purpose of medical care for wounds is to prevent complications and preserve function.

Infection can develop in any type of wound be it surgical, traumatic, accidents e.t.c. The main complication of an open wound is infection. When a wound fails to achieve sufficient healing after 4 weeks of standard care, the wound becomes more painful (instead of gradually improving), look red around the skin edges (this red may feel warm or hot), look swollen, ooze a yellow material (pus) which maybe smelly, the wound can be said to be infected [29]. Chronic wounds such as diabetic foot ulcers, venous leg and pressure wounds to mention but a few, are a challenge to wound care professionals and consume a great deal of health care resources around the globe. Cost for such conditions has been reported to cost 2% or 3% of the health care budgets in developing countries [33].

Treatments of such wounds usually require the use of antibiotics which can be expensive and prolonged use of these antibiotics can lead to the emergence of resistant strains of organisms in the wound. Wound infection can lead to loss of working hours, spending huge amount of money on treatment and can eventually lead to loss of body part and amputation. Cleaning and dressing of wound is a conventional method of treatment that can lead to skin irritation and pain during changing of wound dressing. Bleeding is also another complication from dressing removal and has been reported to cause 6 deaths in 17 injuries [34]. The treatment of the resulting polymicrobial infection has been complicated with the development of resistance to the disinfectants and antibiotics used for wound treatment.

In recent times, there are incidences of preferences to the use of indigenous traditional medicines from natural source (bio disinfectants) for wound care even when conventional medicines are available. This arise due to certain experiences and prejudices that include perceived failure of conventional therapy to meet expectations of the patients especially as regards the management outcomes of such wound conditions as compound bone fracture, chronic ulcers, side effects of drugs highcost of conventional therapy in a typical traditional African approach to wound care. Diverse practices such as the use of herbal medicine, ritual performance and divination are common in African cultures.

Therefore, the interest in the use of natural bio-disinfectant has emerged in recent times because they are readily available in large quantity, effective, less toxic and cheap [17]. This study tends to isolate chitosan from natural source (cockroach and cricket) and evaluate its antimicrobial potential.

2. Materials and Methods

2.1. Collection of Test Organism

Bacterial species, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae and Streptococcus pyogenes were obtained from Microbiology Department, Federal University of Technology, Minna (The organisms were obtained from various wounds of various patients attending General Hospital, Minna and Ibrahim Badamasi Babangida hospital, Minna).

2.2. Collection and Identification of Insects

The *Acheta domisticus* and *Periplaneta Americana* were collected from Bosso area, behind Federal University of Technology, minna. They were taken to the Department of animal biology for authentication by two independent Entomologist. The body of the insects were rubbed with 80-85% alcohol to preserve the body from drying, decaying and breaking into pieces. Then stored in a big jar that has an air tight lid until required.

2.3. Preparation of the Extract

The insects were sorted into cockroach and cricket species and their exoskeleton were prepared for the study by first removing the internal organs and scraping any loosed tissue. The materials were then washed, dried and crushed into powder using mortar and pestle.

2.3.1. Isolation of Chitin

The exoskeleton does not exist as standalone biopolymer, but rather in a conglomeration with other biomaterials, mainly proteins, lipids and inorganic salt. In the process,

treatment of the exoskeleton with 5% HCl dissolved the calcium salts and lipids and other minor inorganic constituents that yielded chitin. Treating the resultant chitin with 40% NaOH solution at 120°C for 2 hours with stirring for homogeneity, yielded the desired chitosan [22, 30].

During the alkaline treatment, a concomitant hydrolysis of the acetamido groups of chitin takes place, this resulted in the formation of Chitosan.

2.3.2. Synthesis of chitosan

The resultant chitosan was washed thoroughly with distilled water and oven dried at 50 °C for 24 hours [7, 27].

The extracts were purified by dissolving in 2% acetic acid and re precipitated out using 20%

NaOH solution. The pure chitosan was then washed to neutrality and oven dried for 24 hours and then grounded into powder with an electrical blender. The process of deacetylation of chitin to chitosan were confirmed using the method of Kumar and Verma [12], 5ml of I_2 / KI solution was added to each testube which gave a yellow colouration to the solution. To this solution concentrated sulphuric acid was added, the colour changes from yellow/brown to dark purple indicated the presence of chitosan. Purification and characterization of chitosan from *P. americana* and *A. domesticus* was previously done [31].

2.4. Determination of MIC and MBC

Only extracts that showed activity against test isolates were used for the MIC. Using tube dilution method, the MIC was determined by serially diluting the extract from 10^1 to 10^{10}. 1ml of each of the dilutions representing a known concentration of the extract was introduced into 9ml of nutrient broth in the test tube. The mixtures were then inoculated with 0.1ml culture of the test organisms standardized to approximately 10^6cfu/ml. This was then incubated at 37°C for 24 hours. The least concentration of the extract in the test tube with no turbidity or cloudiness compared with the control was taken as the MIC [4, 6]. Subsequently those test tubes that showed no turbidity were plated out on nutrient agar plates and absence of growth on incubation for 24 hours confirmed the MBC. The least concentration that showed no visible growth on sub-culturing was taken as MBC. These experiments were done in triplicates.

2.5. Statistical Analysis

The data generated were analyzed by ANOVA and Turkeys post hoc test using spss soft ware.

3. Results

3.1. Antimicrobial Susceptibility Testing

From Tables 1 and 2, it can be seen that the chitosan inhibited the growth of the organisms at various concentrations for each organism as compared to the control (ampiclox becham).

Table 1. *Antibacterial activity of Crude P. americana chitosan against test isolates.*

Isolates	220mg/ml	240mg/ml	Concentrations of extract			Ampiclox
			260mg/ml	280mg/ml	300mg/ml	
			Zones of inhibition (mm)			
Staphylococcus aureus	00.00 ± 00.00^a	3.13±1.12^b	16.67 ± 3.29^d	20.00 ± 1.14^e	20.00 ± 1.14^e	29.35 ± 3.71^g
Bacillus subtilis	3.13±1.14^b	00.00 ± 00.00^a	20.00 ± 1.14^e	20.00 ± 1.14^e	26.01 ± 3.13^f	26.01 ± 3.13^f
Klebsiella pneumoniae	00.00 ± 00.00^a	00.00 ± 00.00^a	00.00 ± 00.00^a	00.00 ± 00.00^a	00.00 ± 00.00^a	28.45 ± 4.65^g
Streptococcus pyogenes	00.00 ± 00.00^a	00.00 ± 00.00^a	13.56 ± 4.21^c	16.67 ± 3.29^d	20.00 ± 1.14^e	00.00 ± 00.00^a
Pseudomonas aeruginosa	00.00 ± 00.00^a	3.00 ± 1.00^b	20.00 ± 3.56^e	20.00 ± 3.56^e	26.01 ± 3.13^f	31.78 ± 3.01^h
Staphylococcus epidermidis	00.00 ± 00.00^a	00.00 ± 00.00^a	00.00±00.00^a	00.00 ± 00.00^a	00.00 ± 00.00^a	28.57 ± 2.48^g

Values are zones of inhibition mean ± standard error of mean triplicate determination. Values with the same superscript in the same column are not significantly different at $p< 0.05$.
mm- millimeter.
Mg/ml – milligram per militer

Table 2. *Antibacterial activity of Crude A. domesticus chitosan against test isolates.*

Isolates	220mg/ml	240mg/ml	Concentrations of 260mg/ml	Extract 280mg/ml	300mg/ml	Ampiclox
			Zones of inhibition (mm)			
Staphylococcus aureus	00.00 ± 00.00^a	12.03 ± 1.05^b	20.00 ± 3.71^d	20.00 ± 3.71^d	20.00 ± 3.71^d	29.35 ± 3.71^g
Bacillus subtilis	00.00 ± 00.00^a	10.05 ± 3.13^b	22.01 ± 3.13^e	22.01 ± 3.13^e	22.01 ± 3.13^e	26.01 ± 3.13^f
Klebsiella pneumoniae	00.00 ± 00.00^a	13.02 ± 11.03^a	15.45 ± 4.65^c	15.45 ± 4.65^c	20.00 ± 3.71^d	28.45 ± 4.65^g
Streptococcus pyogenes	00.00 ± 00.00^a	00.00 ± 00.00^a	00.00 ± 00.00^a	00.00 ± 00.00^a	00.00 ± 00.00^a	00.00 ± 00.00^a
Pseudomonas aeruginosa	12.03 ± 10.00^b	15.45 ± 4.65^c	22.78 ± 3.01^e	22.78 ± 3.01^e	26.01 ± 3.13^f	31.78 ± 3.01^h
Staphylococcus epidermidis	00.00 ± 00.00^a	00.00 ± 00.00^a	17.57±2.48^c	17.57±2.48^c	20.00 ± 3.71^d	28.57 ± 2.48^g

Values are zones of inhibition mean ± standard error of mean triplicate determination. Values with the same superscript in the same column are not significantly different at $p< 0.05$.
mm- millimeter. Mg/ml – milligram per milliter.

3.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC of *P. Americana and A. domesticus* Crude on test isolates are shown in tables 3 and 4. The MIC for *P. Americana* Crude against *B. subtilis, P. aeruginosa, S. aureus, S. epidermidis, K. pneumoniae* and *S. pyogenes* were 240, 220, 0, 260, 240 and 0 mg/ml while MBC were 260, 260, 0, 280, 260 and 0mg/ml respectively.

The MIC for *A. domesticus* Crude against *B. subtilis, P. aeruginosa, S. aureus, S. epidermidis, K. pneumoniae* and *S. pyogenes* were 240, 240, 240, 0, 220 and 280 mg/ml while the MBCs were 240, 240, 280, 0, 240 and 300 mg/ml respectively.

Table 3. *Minimum Inhibitory Concentration of Crude chitosan of P. americana and A. domesticus against test isolates.*

Isolates	MIC of Extract Concentration (mg/ml)	
	Periplanata americana	***Acheta domesticus***
Staphylococcus aureus	240	240
Bacillus subtilis	220	240
Klebsiella pneumoniae	0	240
Streptococcus pyogenes	260	0
Pseudomonas aeruginosa	240	220
Staphylococcus epidermidis	0	280

mg/ml: milligram per millitre MIC: Minimum Inhibitory Concentration

Table 4. *Minimum Bactericidal Concentrationof Crude P. americana and A. domesticus chitosan against test isolates.*

Isolates	MBC of Extract Concentration (mg/ml)	
	Periplaneta americana	***Acheta domesticus***
Staphylococcus aureus	260	240
Bacillus subtilis	260	240
Klebsiella pneumoniae	0	280
Streptococcus pyogenes	280	0
Pseudomonas aeruginosa	260	240
Staphylococcus epidermidis	0	300

mg/ml: milligram per millitre MBC: Minimum Bactericidal Concentration.

4. Discussion

Antibacterial activity of Crude Chitosan of *Periplanata Americana* produced 26.01±3.13mm diameter zone of inhibition against *B. subtilis* and *P. aeruginosa*. This implies that the organisms were most sensitive to the Crude Chitosan of *P. americana*. The antibacterial activity of Crude Chitosan of *P. americana* produced 20.00±1.14mm diameter zone of inhibition against *S. aureus* and *S. pyogenes.* This implies that the organisms were sensitive to the extract. Umar *et al*. [31] reported that the extract contained metabolites like benzene derivative, fatty acid, alkene derivative, bicyclo derivative, bicyclo alcohol derivative, alkane derivative, saturated fatty acids, unsaturated alcohol derivative, saturated tricyclo derivative, unsaturated bicyclo derivative, chloro alkane derivative, saturated alcohol, unsaturated alcohol and multiple cyclic compounds when Gas Chromatography – Mass Spectrometery was carried out on the extracts. These compounds have been reported to have antibacterial activity [18]. The diameter zone of inhibition of 00.00±00.00mm was produced by the extract against *K. pneumoniae* and *S. epidermidis.* This implies that the organisms were resistant to the extract.

Ampiclox becham was the control, it produced 31.78±3.01mm diameter zone of inhibition against *P. aeruginosa*, and this implies that the organism was most sensitive to the drug. Ampiclox produced 29.35±3.71 mmdiameter zone of inhibition against *S. aureus* this implies that the organism was also sensitive to the drug. Ampiclox produced 28.57±2.48 and 28.45±4.65mm diameter zone of inhibition against *S. epidermidis* and *K. pneumonia*e, this implies that the organisms were sensitive to ampiclox. Ampiclox produced 26.01±3.13mm diameter zone of inhibition against *B. subtilis* which implies that the organism was sensitive to the drug. 00.00±00.00mm diameter zone of inhibition was produced against *S. pyogenes*, this implies that the organism was resistant to the drug. Resistance of *S. pyogenes* could be due to intra cellular persistence of the organism, due to poor penetration of penicillin in to tonsular tissues or protection of *S. pyogenes* by beta lactamase producing bacteria (*S. aureus*) or coaggregation between organism like *M. catarrhalis* and *S. pyogenes* which may enhance *S. pyogenes* colonization through the facilitation of its adherence to human epithelial cells or the alteration of the commensal bacterial microbiota which can compete for nutrients [2, 24]. Resistance of organisms to ampiclox is in line with the findings of Jose *et al*. [10], who reported that *S. pyogenes* isolated from wounds were resistant to ampiclox, penicillin and some other antibiotics.

Antibacterial activity of Crude *Acheta domesticus* produced 26.01±3.13mm diameter zone of inhibition against *P. aeruginosa*. This implies that the organism was most sensitive the extract. 22.01±3.13mm diameter zone of

inhibition was produced against *B. subtilis*, which implies that the organism is sensitive to the extract. Antibacterial activity of Crude *A. domesticus* produced 20.00±3.17mm diameter zone of inhibition against *S. aureus*, *K. pneumoniae* and *S. epidermidis*. It implies that the organism was sensitive to the extract. The extract contained metabolites like benzene derivative, fatty acid, saturated tricyclo derivative, saturated fatty acid alkene derivative, bicyclo derivative, bicyclo alcohol derivative, unsaturated alcohol derivative, chloro alkane and multiple cyclic compounds which could be a reason for its antibacterial activity [31]. 00.00±00.00mm diameter zone of inhibition against *S. pyogenes,* this implies that the organism was resistant to the extract.

The highest zone of inhibition for the crude extract was 20.00± 3.56mm for *P. Americana* and for *A. domesticus* was 22.78±3.01mm. Clinical and Laboratory Standards institute (CLSI) protocol [4] on antibacterial susceptibility testing states that extracts that have the zones of inhibition of 14mm and above have significant antimicrobial activity. The crude extract of *P. americana* and *A. domesticus* had zones of inhibition over 14mm, which implies that they have significant antimicrobial activity.

The crude extract had activity (killed) against *S. aureus* after 24 hours of exposure, this could be due to the fact that it has simple bilayer cell structure, the mechanism of action should be related to the fact that the derivative has strong coordination capability and its amphiphilic structure [32].

The work of Jianhui and Shaoling, [9] states that chitosan and its derivatives show antibacterial activity against fungi, gram positive bacteria and gram negative bacterial. Their work clearly analyzed the effects of Molecular weight, chemical modification, solubility, and chitosan based composites on antibacterial properties and mechanism of chitosan. Prashik *et al*. [26] determined the MIC and MBC of silver nanoparticles against *S. aureus* and obtained low MIC (0.625mg/ml), which is similar to the result obtained in this study.

The MIC obtained in this study is similar with the work of Nkechinyere *et al*. [23] who obtained a similar range of MIC after antibacterial susceptibility testing of chitosan on *Staphylococcus* and *Streptococcus species.* The work of Azam *et al*. [1] also recorded low MIC when they tested chitosan on *Streptococcus* specie, chitosan reduced biofilm formation in the organism. The chitosan isolated from *A. domesticus* and *P. Americana* had MIC and MBC which implies that the chitosan had bactericidal and bacteriostatic effects on test isolates.

The study of Muhammed *et al*. [21] focused on the biological activities of chitosan products with a viscosity average molecular weight ranging from 22 to 846kDa in combination with the most active monoterpenes against four bacteria, *Pseudomonas* inclusive indicating a similar result with that of this study. The MIC was found to be dependent on the type of the microorganism tested.

5. Conclusion

This study reveals that chitosan from cockroach and cricket have potential bactericidal effects against *S. aureus, S. epidermidis, K. pneumoniae, S. pyogenes, B. subtilis* and *P. aeruginosa* at the concentration of 220mg/ml which is lower than synthetic disinfectants customarily used. The principle underlying the development of tube dilution method is that bacteria made dormant (but not killed) by a particular concentration of antibiotic can be revived if that antibiotic concentration is remarkably reduced by dilution, while bacteria killed by antibiotic at a given concentration cannot be revived no matter the dilution. Chitosan from cockroach and cricket have both abilities since they have MIC and MBC hence they are fit to be incorporated in wound dressing medicaments (applied topically). The chitosan isolated from *A. domesticus* and *P. Americana* had MIC and MBC which implies that the chitosan had bactericidal and bacteriostatic effects on test isolates.

Conflict of Interest

All the authors do not have any conflict of interest.

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