Evaluation of the Antibacterial and Antifungal Activities of Chitosan Prepared from the American Cockroach, (Periplaneta americana)

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ABSTRACT
Microorganisms such as bacteria and fungi are developing resistance to the current therapies easily and the presently available antibacterial, antifungal agents and pesticides are certainly very pricey and harmful. So, the necessary shift to use natural antimicrobial extract (Periplaneta americana chitosan) seems to be more effective, economic and safe to the public health. The American cockroach chitosan was dissolved in 1 % acetic acid and diluted to different concentrations then, seeded in 96 well tissue culture plates to evaluate the antibacterial activity by using MTT assay and the microbial growth was calculated by ELISA microplate reader. The American cockroach chitosan showed an equal minimum inhibitory concentration (MIC) antibacterial action against two (Gram-positive) bacterial strains Staphylococcus aureus, and Bacillus subtilis, recording MIC = 2000 µg/ml. Also, chitosan showed MIC antibacterial activity against Escherichia coli and Salmonella typhimurium (Gram-negative) bacteria recording MIC = 1000 µg/ml, and MIC = 2000 µg/ml respectively. While chitosan showed non-antifungal activity against Candida albicans. Natural chitosan could be considered as a determined factor affecting on the biological activities mentioned in this study.

INTRODUCTION
The increasing complication of antibiotic resistance by microbial organism's requests to research for new natural compounds, so insects have attracted the concern of entomologists for several reasons regarded as availability, widely and biodiversity of its species. The bioactive effect of some proteins that have been characterized from insects, with antimicrobial effects, were fascinating scientists for their potential benefits. However, recent improvement in biotechnology helped scientists to evaluate the antimicrobial activity of the insect's extracts (Ratcliffe et al., 2011). Cockroaches are capable of acquiring and infecting other cockroaches and objects, therefore implicating them as potential vectors of food borne pathogens in poultry production and processing facilities (Kopanic et al. 1994).
The insect immune responses based on the knowledge of the pathogen as nonself, farther on the induction of suitable genes and biochemical pathways appear in the manufacture of a potent arsenal of antibacterial ingredient (Seufi, et al., 2011).

Insects (cockroaches) protected themselves from pathogens and parasites via a powerful innate immune system, as cuticle immune, which contains antimicrobial agents (Pai et al. 2004). The chitin extracted from cuticle and chitosan of the cockroach (Periplaneta americana), are a versatile material with effectiveness in reducing microorganism growth and multiplication (Goy et al. 2009; Kim, et al. 2017). Insect extracts showed antimicrobial activities used universally in medicine for managing many diseases (Feng et al., 2009), also ingredients isolated from insects indicated antifungal activity against multi pathogenic fungi (Tomie et al., 2003). Chitosan, a natural antimicrobial agent, kept antimicrobial activity in different matrices and that provided a promising solution to enhance animal and public health (Ma, 2017). Chitosan obtained from chitin exhibited excellent antimicrobial activities against many species of Gram-positive and Gram-negative pathogenic bacteria (Chien et al., 2016). Chitosan was characterized by its highly microbicidal activities (Balicka-Ramisz et al., 2005).

The purpose of this research was to investigate the antibacterial and antifungal activities of Periplaneta americana chitosan, against two (Gram-positive) bacteria namely, Staphylococcus aureus and Bacillus subtilis, and two different (Gram-negative) bacteria, Salmonella typhimurium and Escherichia coli, and a single fungal model, Candida albicans.

MATERIALS AND METHODS

1. The Antimicrobial Activity of Chitosan:
The American cockroach chitosan was prepared and characterized with a degree of deacetylation (DDA) above 50% according to Mahmoud et al., (2021). Chitosan was dissolved in 1 % acetic acid (v/v) and diluted to a concentration of 8 mg/ml, with 1:2 serial dilutions to reach concentrations ranging from 8000 to 3.9 μg / ml. A quantity of 5 μl of each dilution was seeded in 96 well tissue culture plates, as well as a sterility control and growth control. Each test and growth control well was inoculated with 5 µl of microbial suspension (10^5 CFU/well). 10 µl methyl-thiazolyldiphenyl-tetrazolium (MTT) (Mosmann 1983) (5 mg/ml) was dispensed to each well and the plates were incubated for 3 h at 37 °C, 50 μl of the Dimethyl sulfoxide (DMSO) solution was added to these wells at room temperature for 30 minutes, then it was readed using ELISA microplate reader at 750 nm, then the microbial growth was calculated by equation.

\[
\text{Microbial growth} \% = \frac{\text{OD of treated test}}{\text{Mean OD of growth control}} \times 100
\]

OD → Optical density.

2. The Antibacterial Activity:
The antibacterial activity was evaluated by the minimum inhibitory concentration (MIC) method, according to the method of Souza et al., (2005). All strains were tested and obtained from The Laboratory of Microbiology, Botany and Microbiology Department, Faculty of Science (Boys), Al-Azhar University, Nasr City, Cairo, Egypt. Four pathogenic bacterial strains were used for the antibacterial assay belonging to two groups:

- Gram-positive bacteria: Staphylococcus aureus, Bacillus subtilis
- Gram-negative bacteria: Escherichia coli, Salmonella typhimurium

The microorganisms were growing in nutrient agar medium consisted of: peptone 5.0 gm/L; beef extract 3.0 gm/L; and agar-agar 15.0 gm/L. the pH was adjusted to 7.0 before sterilization (Tadashi 1975).
3. The Antifungal Activity:
The antifungal activity was evaluated by the minimum inhibitory concentration (MIC) method, according to the method of Souza et al., (2005). The single fungal strain (Candida albicans) was obtained from The Laboratory of Microbiology, Botany and Microbiology Department, Faculty of Science (Boys), Al-Azhar University, Nasr City, Cairo, Egypt.

The microorganism was growing in a sucrose-nitrate agar medium consisted of sucrose 30 gm/L; NaNO₃ 2.0 gm/L; K₂HPO₄ 1.0 gm/L; MgSO₄. 7H₂O, 0.5 gm/L; agar 15.0 gm/L and distilled water 1000 ml. The pH value was adjusted at 7-7.3 before sterilization (Tadashi 1975).

**RESULTS**
**1. Evaluation of the Antibacterial Activity of Chitosan:**
**I. The Antibacterial Activity of the Chitosan Against Gram-Positive Bacteria:**
Chitosan with different concentrations was tested for its antibacterial activity, data arranged in table (1) by using the minimum inhibitory concentration (MIC) assay, and illustrated in figure (1). The antibacterial (growth- inhibitory) activity of chitosan after 24h (post-treatment) against Staphylococcus aureus showed complete inhibition percentage (100%) at concentrations of; 8000, 4000 and 2000µg/ml, and decreased gradually to the lowest concentrations until no inhibition recorded at a concentration of 31.25 µg/ml.

Also, chitosan showed 100% inhibition activity against Bacillus subtilis at concentrations of; 8000, 4000 and 2000µg/ml, while it decreased sequentially to the lowest concentrations until no inhibition recorded a concentration of 15.63µg/ml. From the results, it was obvious that chitosan recorded minimum inhibitory concentration antibacterial activity of 2000 µg/ml against the two tested Gram-positive bacterial strains.

**Table 1:** Evaluation of P. americana chitosan antibacterial activity against Gram-positive bacteria using the MIC assay.

<table>
<thead>
<tr>
<th>Chitosan concentration µg/ml</th>
<th>Staphylococcus aureus</th>
<th>Bacillus subtilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>8000</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4000</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2000</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1000</td>
<td>86.94</td>
<td>84.25</td>
</tr>
<tr>
<td>500</td>
<td>70.36</td>
<td>66.96</td>
</tr>
<tr>
<td>250</td>
<td>52.2</td>
<td>52.71</td>
</tr>
<tr>
<td>125</td>
<td>38.05</td>
<td>41.93</td>
</tr>
<tr>
<td>62.5</td>
<td>8.22</td>
<td>18.09</td>
</tr>
<tr>
<td>31.25</td>
<td>0</td>
<td>6.74</td>
</tr>
<tr>
<td>15.63</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.81</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.9</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
II. The Antibacterial Activity of Chitosan Against Gram-Negative Bacteria:

As shown in table (2) and figure (2) the antibacterial (growth- inhibitory) activity of chitosan after 24h post-treatment against *Escherichia coli* showed complete inhibition percentage (100 %) at concentrations of 8000, 4000, 2000 and 1000 µg/ml, and decreased gradually at the lowest concentrations until no inhibition recorded at concentration of 3.9 µg/ml.

On the other hand chitosan showed 100 % inhibition activity against *Salmonella typhimurim* at concentrations of; 8000, 4000 and 2000µg/ml, while it decreased sequentially to the lowest concentrations until no inhibition recorded at 7.81µg/ml. From the results, it was obvious that chitosan showed acceptable antibacterial activity against the two different (Gram-negative) bacterial strains. The minimum inhibitory concentrations were 1000 µg/ml and 2000 µg/ml for *Escherichia coli* and *Salmonella typhimurim*, respectively.

Table 2: Evaluation of the American Cockroach chitosan antibacterial activity against Gram-negative bacteria using the MIC assay.

<table>
<thead>
<tr>
<th>Chitosan concentration µg/ml</th>
<th><em>Escherichia coli</em></th>
<th><em>Salmonella typhimurim</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>8000</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4000</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2000</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1000</td>
<td>100</td>
<td>86.556</td>
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<tr>
<td>500</td>
<td>91.95</td>
<td>67.71</td>
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<td>125</td>
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<td>31.756</td>
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<td>9.01</td>
</tr>
<tr>
<td>7.81</td>
<td>7.72</td>
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</tr>
<tr>
<td>3.9</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Evaluation of the Antibacterial and Antifungal Activities of The American Cockroach Chitosan

Fig. 2: The antibacterial activity of American Cockroach chitosan against Gram-negative bacteria by using the MIC assay.

2. The Antifungal Activity of Chitosan:
Chitosan with different concentrations was tested for its antifungal activity against *Candida albicans*, by using the MIC assay. Non-antifungal (growth-inhibitory) activity of chitosan was recorded after 24h post-treatment.

DISCUSSION
In the present study, promising results of the American cockroach chitosan against *Staphylococcus aureus* (Gram-positive) bacteria were showed minimum inhibitory concentration in covenant with results of Gerasimenko et al., (2004) where they found that bee chitosan also suppressed the growth of *S. aureus* at concentrations of 0.5 and 0.25%, likewise İlk et al., (2020) showed that (Gram-positive) bacteria *S. aureus* inhibited by chitosan membrane obtained from insect corneal lenses of *Tetanus* bovines. While on the conflict with our results, the chitosan synthesized from fish scales, crab and shrimp shells didn’t show any activities against *S. aureus* according to Kumara et al., (2017).

The American cockroach chitosan showed decrees in the bacterial growth of *Bacillus subtilis*, Chandrasekaran et al., (2020) and Tikhonov et al., (2006) results attained similar where they found that chitosan itself possesses antimicrobial activity against many Gram-positive bacteria as *B. subtilis*. In addition, No et al., (2002) explored that chitosan with different molecular weights possesses antibacterial activity against Gram-positive bacteria (*Bacillus* sp).

Chitosan showed strong inhibitory activity against *Escherichia coli* after 24h post-treatment. In agreement with Divya et al., (2017) chitosan has antibacterial activity, against Gram-negative bacteria, *E. coli* with minimum inhibitory concentration (10% MIC). In addition, Fei Liu et al., (2001) observed an effective antibacterial activity of chitosan against *E. coli* with (20% MIC) depending on the molecular weight (MW) of chitosan. On the other hand, Shin, et al., (2019) disagreed with our results, where they found that chitosan from the mealworm beetle showed non-antibacterial activity against *E. coli* by using the MIC assay.

From results it was obvious that the American cockroach chitosan showed potent antibacterial activity against Gram-negative bacteria *Salmonella typhimurium*, in covenant with these results, (Tsai et al., 2002) illustrated that chitosan obtained from shrimp shell showed antibacterial action against *S. typhimurium*. Similarly with the results of Balicka-Ramisz et al., (2005) the effectiveness of crab chitosan solution against *Salmonella* sp, showed powerful,
effectiveness and inhibitory activity against the bacterial strain with 2 MIC.

On the other hand, our results showed non-antifungal activity against Candida albicans, hassling with these results Basseri et al., (2019) tested the antifungal activity of chitosan against Candida albicans where they noted that chitosan showed non-antifungal activities. In the opposite of Alburquenque et al., (2010) effect of chitosan against clinical Candida sp isolates exhibited significant antifungal activity.

In general, it was obvious that chitosan possesses antibacterial activity against tested bacteria, with no action against Candida albicans, in line with Shahraki et al., (2018) extracted chitosan showed different degrees of antibacterial activity against both Gram-positive and Gram-negative bacteria such as S. aureus, E. coli, and P. aeruginosa. In addition, chitosan did not show any activity against C. albicans fungi.

CONCLUSION

Chitosan is a natural polysaccharide commonly found as a constituent of the cuticle of insects, as in the American cockroach. It has a biological role as an antibacterial and antifungal agent. The present results showed promising antibacterial activities of the American cockroach chitosan against the tested (Gram-positive) bacteria namely; Staphylococcus aureus and Bacillus subtilis, while chitosan showed reasonable antibacterial activities against both tested (Gram-negative) bacteria; Escherichia coli and Salmonella typhimurium. On the other hand, chitosan didn’t show any antifungal activity against Candida albicans.

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