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Evaluation of beetles Scarabaeus Sacer Derived-Chitosan, Anti-Cancer and Anti-bacterial Potentials: In Vitro Study

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Lung and colorectal cancer represent a major health problem all over the world, in addition, bacterial infections impose a serious medical and health concern. Thus, in this study, we evaluate the cytotoxic effect, anti-cancer and anti-bacterial properties of beetles derived chitosan. The study conducted using lung (A549) and colorectal (HCT-116) cancer cell lines to identify the anti-cancer effect and antimicrobial activity against gram-positive bacteria (*Streptococcus pyogenes*, *streptococcus Aureas*) and gram-negative bacteria (*Salmonella typhimurium*). Cytotoxicity was evaluated by describing and measuring recoding morphological changes. The viability and related IC50 were cell type and concentration dependent. Also, related cell apoptosis was monitored using PI stain where early and late apoptosis of treated A549 cells was significantly elevated than in case of HCT-116 cell line (P<0.05). In the meantime, the necrosis % of treated cells didn't perform any changes between the two cell lines but significantly elevated than that of cell control (P<0.05). Apoptotic profile was examined and showed up regulation of both pro-apoptotic genes (P53 and Bax) accompanied with down regulation of BCL-2 in both cell lines. Also, gram positive bacteria showed a remarkable enhancement by beetles' chitosan unlike gram negative bacteria that showed a lower respond to chitosan anti-microbial property.

Keywords: Beetles' chitosan, toxicity, cell cycle, apoptosis, anti-cancer, anti-bacterial.

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INTRODUCTION

Cancer is a leading cause of death in both economically and developing countries (Torre et al., 2015). Cancer is a group of diseases that developed abnormally and invade throughout the body that may be caused by many causes like excessive exposure to sunlight or to ionizing Radiation, obesity, smoking, Poor life style, lack of physical activity, environmental pollutants, drinking large amount of alcohol & other infections such as Hepatitis B, C (WHO,2018). Colorectal cancer (CRC) is the third most common cancer worldwide after lung and breast cancers with two-thirds of all colorectal cancers occurring in the more developed regions of the world (bowel, 2011; Dragovich & Tsikitis, 2012). In Egypt, Colon cancer is uncommon and represent only 3 percent of malignant

tumors (excluding nervous system cancer patients) (sheriff & ibrahim, 1987). Population based studies as well as hospital-based studies in Egypt and Arab countries have shown high Colorectal cancer rates in patients aged 40 years or younger (Al-ahwal&Al-ghamry,2005; Abdullah, Musa & kheir, 2007; Soliman et al, 1998; Khafagy et al, 2000 and Samer et al., 2009). Diet and lifestyle factors are implicated risk factors for the disease. Fruit and vegetable-deficient diet, calorie-dense foods, physical inactivity, obesity, and smoking increase risk for developing colorectal cancer(Gado, Ebeid, Abdel Mohsen, & Axon, 2014).Lung cancer is the most common cancer in worldwide, around 1.8 million people are diagnosed with lung cancer every year, 1.6 million people died from it (WHO, 2017). In Egypt, there were an estimated 5,017 lung cancer cases and an estimated 4,488 lung cancer death in 2012. (GLCC, 2017) Cigarette smoking causes most lung cancers. The more cigarettes smoked per day and the earlier the age at which one started smoking, the greater the risk of lung cancer. High levels of pollution, radiation and asbestos exposure may also increase the risk. (DePerrot, Licker & Bouchardy, 2018). Chitosan is one of the most common natural polymers that can be obtained from various species, particularly from the exoskeletons of crustaceans. It is also found in cuticles of insects as well as in the cell walls of fungi and some algae (Sandford and Hutchings, 1987; EPA, 1995). Chitosan is a polysaccharide derived from a low acetyl form of chitin, mainly composed of glucosamine and N-acetylglucosamine. Its structure and composition are similar to both cellulose and chitin (Freepons, 1991; Hadwiger and McBride, 2006). Chitin has low toxicity and is inert in the gastrointestinal tract of mammals; it is bio degradable, owing to the presence of chitinases widely distributed in nature and found in bacteria, fungi and plants, and in the digestive systems of many animals. However, the main development of chitin film and fiber is in medical and pharmaceutical applications as wound-dressing material and con-trolled drug release. Chitin is also used as an excipient and drug carrier in film, gel or powder form.(Rinaudo, 2006). In this study, Beetle's-derived chitosan is used to evaluate its anticancer and antimicrobial potentials against A549 , HCT-116 , Gram positive and negative bacterial strains

MATERIALS

All the materials of Human lung carcinoma were obtained from the American type culture collection (ATCC-A549) and Human colorectal carcinoma, (HCT-116) cell lines and beetles-derived Chitosan from Scarabaeus sacer were kindly supplied from International Center for Advanced researches [ICTAR-Egypt]. Chitosan was sterilized using Ethylene oxide gas (C2H4O) at 52oC for 6 hrs. Cell lines were maintained according to the standard recommended instructions. Cell culture medium, trypsin and Ethylenediaminetetraacetic acid (EDTA) and Fetal Bovine serum (FBS) were kindly purchased from [GIBCO-USA]. While, Cell culture plates and flasks were purchased from [TPP-Swiss].

METHODS

Cytotoxicity assay

Cancer cells were propagated in 75 Cm2 surface area cell culture flasks using Roswell Park Memorial Institute medium (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS) and incubated in 5% CO2 incubator at a temperature of 37°C. Confluent cells were detached using 0.25% trypsin solution and 0.25% ethylene diamine tetraacetic acid (Gibco-USA) for 5 min. Cells were plated at a concentration of 2 x 105 cells / ml in 96-well cell culture plates and incubated at a temperature of 37°C for 24 hours to achieve confluency. The medium was decanted and fresh medium containing twofold serially diluted concentrations of beetles' chitosan was added post dissolving in 1% acetic acid for cytotoxicity determination using colorimetric MTT reduction assay. Dead cells were washed out using phosphate-buffered saline (PBS), and 50 µl of MTT stock solution (5 mg/ml) were added to each well. After 4 hrs. incubation, the supernatants were discarded and the formazan precipitates were solubilized by addition of 50 µl / well of dimethyl sulfoxide (DMSO). Plates were incubated in the dark for 30 min at a temperature of 37oC, and absorbance was determined at a wavelength of 570 nm using microplate reader (ELX-800, Biotek, USA) (Ciapetti, Cenni, Pratelli, & Pizzoferrato, 1993). The cell viability percentage was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Mean OD of treated wells}}{\text{Mean OD of control well}} \times 100$$

The cell viability (%) was plotted against the tested Chitosan concentrations. The IC50 values of test chitosan were determined using Masterplex-2010 software program. The effect of Beetles' chitosan as anticancer synergistic agent was examined by evaluating the cytotoxic potential of Chitosan in A549 and HCT-116 cell lines. Cells pretreated with IC50 concentration of chitosan for 24 hrs. Morphological alterations of cells were analyzed using an inverted microscope (Nikon-Japan).

Cell cycle analysis

A549 and HCT-116 cells pre-cultured in 25 cm2 cell

culture flasks were treated with the IC50 of tested chitosan dissolved in RPMI-1640 medium, for 24h. For cell cycle analyses, the cells were harvested and fixed gently with 70% ethanol in PBS, maintained at temperature of 4˚C overnight and then re-suspended in PBS containing 40 µg/ml PI and 0.1 mg/ml RNase and 0.1% Triton X-100 in a dark room. After 30 min at 37˚C, the cells were analyzed using a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at a wavelength of 488 nm. The cell cycle and sub-G1 group were determined and analyzed, as described previously.

mRNA Expression levels of cell apoptosis-related genes:

Total RNA was extracted from control and treated A549 and HCT-116 cells using the Gene JET RNA Purification kit (Fermantus-UK) according to the manufacturer's protocol. The concentration and the integrity of RNA were assessed spectrophotometrically at 260/280 nm ratio and by gel electrophoresis, respectively. First-strand of cDNA was synthesized with1 μg of total RNA using a Quantitect Reverse Transcription kit (Qiagen, Germany) in accordance with the manufacturer's instructions. These samples were subsequently frozen at a temperature of -80◦C until usage for determination of the expression levels of P53, Bax and Bcl-2 genes using real-time PCR.Quantitative Realtime PCR was performed on a Rotor-Gene Q cycler (Qiagen, Germany) using QuantiTect SYBR Green PCR kits (Qiagen, Germany) and forward and reverse primers for each gene. The nucleic acid sequences of the primers were as follows : P53 (F: 5'-TCA GAT CCT AGC GTC GAG CCC-3' & R: 5'-GGG TGT GGA ATC AAC CCA CAG-3') and Bax(F: 5'-ATG GAC GGG TCC GGG GAG CA-3' & R: 5'-CCC AGT TGA AGT TGC CGT CA-3') as well as anti-apoptotic gene Bcl-2 (F: 5'-GTG AAC TGG GGG AGG ATT GT-3' & R: 5'-GGA GAA ATC AAA CAG AGG CC-3') compared to β-actin as a housekeeping gene (F: 5'-CAA GGT CAT CCA TGA CAA CTT TG-3' & R: 5'-GTC CAC CAC CCT GTT GCT GTA G-3'). Real-time PCR mixture consisted of 12.5 μL 2x SYBR Green PCR Master Mix, 1 μL of each primer (10 pmol/μL), 2 μL cDNA, and 8.5 μL RNase-free water in a total volume of 25 μL. Amplification and cycles were done as follow: A temperature of 95◦C for 15 min for the initial activation, followed by 40 cycles of denaturation at 94◦C for 15 s, annealing at 60◦C for 30 s, and elongation at 72◦C for 30 s. Melting curves were performed after real-time PCR to demonstrate the specific amplification of single products of interest. A standard curve assay was performed to determine the amplification efficiency of the primers used. Relative fold changes in the expression of target genes (P53, Bax and Bcl-2) were accomplished using the comparative 2−ΔΔCt method with the β-actin gene as an internal control to normalize the level of target gene expression. ΔΔCT is the difference between the mean ΔCT (treatment group) and mean ΔCT (control group), where ΔCT is the difference between the mean CT gene of interest and the mean CT internal control gene in each sample. Logarithmic transformation was performed on fold change values before being statistically analyzed, using the fold change values of three replicates for each gene measurement.

Anti-bacterial effect using CFU (colony forming unit) count:

Staphylococcus Aureas, Staphylococcus Pyogen & salmonella Typhimurium had been obtained by Dr. Aly Fahmy Mohamed from (ICTAR, Egypt). Colonies counted by colony-forming units (CFU) technique. For anti-bacterial analysis, aliquots of 1 ml of bacterial broth of each microorganism were added to 9 ml of chitosan diluted suspensions and kept under moderate shaking at room temperature. The turbidity was measured in each polymeric sample solutions by adding to the mixture of the cultured bacteria medium and PBS (Phosphate-buffered saline), pH 7.2 ±0.2.

Anti-bacterial activity using inhibition zone method:

The plate diffusion method (Ryan et al., 1996), was also used to visualize the formation of a clear zone of inhibition in a TSB (tryptic soy agar) solid culture medium. The procedure carried used in this analysis follows the agar diffusion method, where small circular cavities were punctured in the culture medium and filled with approximately 0.05 ml of dissolved chitosan concentrations. 50 µl of bacterial suspension were spread and the plates stored for 24 h at 37 °C to allow microorganism growth. Inhibition zones were measured on bases of the average diameter of the clear areas, directly on the dishes. Test was performed in triplicate (Abbas and Dutta, 2009).

Statistical analysis

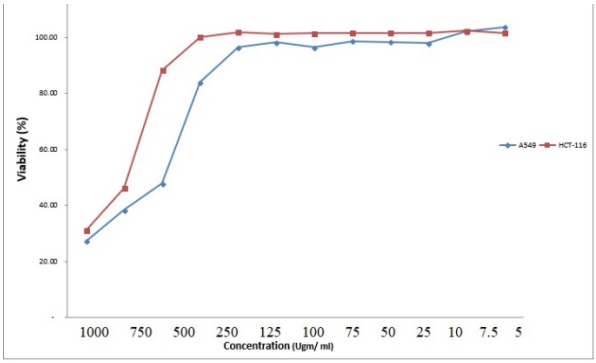
Statistical significance between treated and untreated cells was determined using one-way ANOVA. Differences at P values less than 0.05 were considered significant.

RESULTS

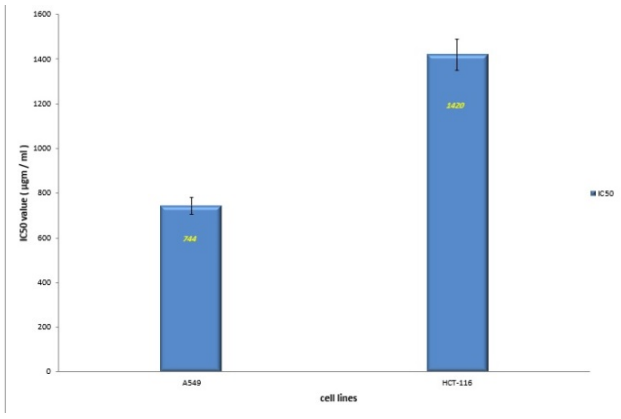
Regarding the toxicity of chitosan, it was noticed that chitosan induced a cell rounding (A549 and HCT-116), surface detaching and membrane rupture. Also, it was

clear that cell viability and IC50 values were concentration and cell type dependent [Figure 1-2]. Also, the pro and antiapoptotic genes were estimated post cell treatment recording upregulation in both P53 and Bax. While, downregulation of Bcl2 occurred in comparison with their values in both cell lines [Figure 3]. Cell toxicity was accompanied with cell apoptosis that was cell type dependent and there was a significantly elevated the percentage of early and late apoptosis in A549 cell line compared with apoptosis profile of untreated cell control. In the meantime, the necrotic % of treated cells didn't perform any change between the two cell lines but compared with its values in cell control there was a significant change ($P<0.05$) [Figure 4-5]. Also, antimicrobial potential was monitored recording those Gram-positive bacteria elected strains were significantly affected by derived chitosan than gram-negative bacteria [Figure 6].

Viability of cells

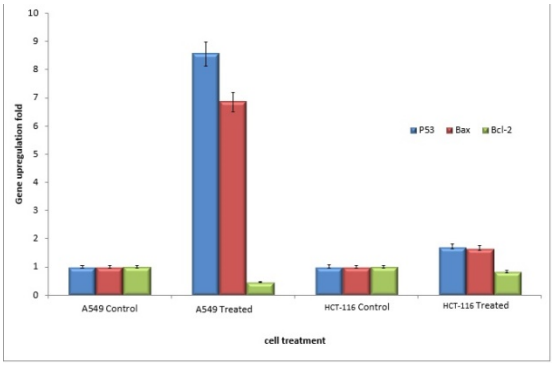


[**Figure 1**]: Evaluation of cell viability after treatment with beetles derived chitosan using MTT Assay relative to concentration As, the viability of cells decreased by increasing the concentration of beetle's chitosan, cell viability decreased to 25%by increasing the concentration of chitosan by 1000 µg/ml in HCT-116 cell lines while reaching 30% in A549 cells



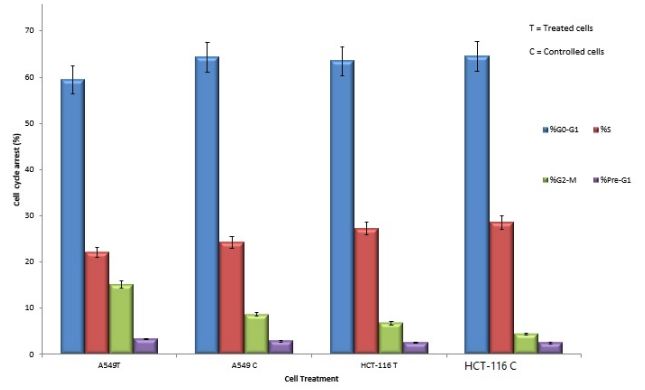
[**Figure 2**]: The IC_{50} values for both A549 and HCT-116 cell lines in both cells using beetles-derived chitosan.HCT-116 cell line shows a higher IC50 value unlike A549 that showed a stringer inhibitory effect

Cell treatment for upregulations and downregulations of genes:



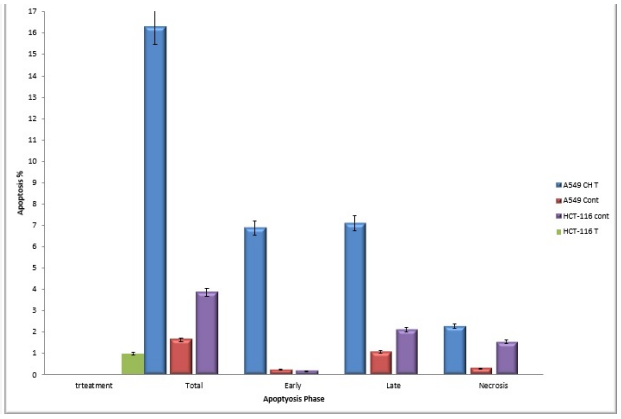
[Figure 3]: Evaluation of up-regulation and down regulation of pro and antiapoptotic genes under the effect of beetles derived chitosan. Pro apoptotic genes were significantly up regulated and anti-apoptotic gene was down regulated in significant way to control cells. Apoptotic profile of A549 was more sensitive to that in case of HCT-116 treanteatme

Cell cycle arrest results:



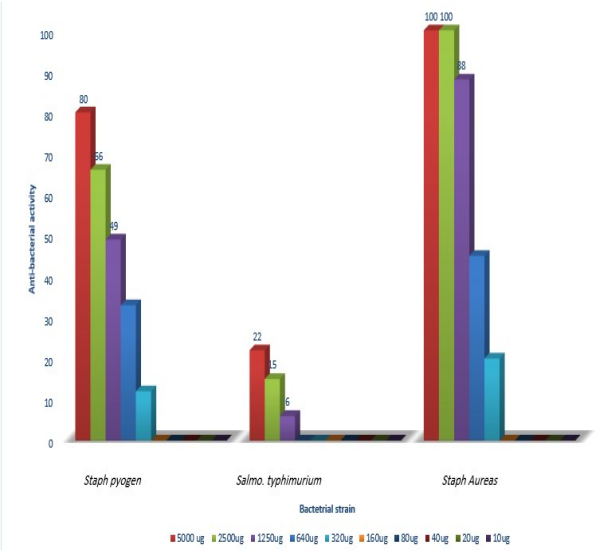
[**Figure 4**]: Cell cycle arrest assay of treated and control A549 and HCT-116 Cell lines. G2-M phase showed variation between treated and untreated

Apoptosis phase and it's percentage:



[Figure 5]: Evaluation of apoptotic and necrotic profile and related changes, As A549 cell line showed a significant early and late apoptosis than HCT-116 While necrosis phase showed non-significant changes in both cell lines

Anti-microbial effect:



[**Figure 6**]: Anti-microbial effect of beetle's derived Chitosan against gram-positive (*pyogen* and *aureas*) and gram-negative bacteria (*salmonella typhimurium*). gram positive bacteria were more sensitive to derived chitosan than gram negative bacteria

DISCUSSION

Anti-cancer mechanism of many anti-cancer agents from natural sources with minimal toxicity for normal cells are still being performed. Chitosan is of the most common example derived from crustacean, insects. Chitin derived chitosan proved to be of anticancer potential. In Vitro studies showed that chitosan had a promising effect on cancer cell lines in both HCT-116 and A549 and showed toxicity to cancer cell lines in a time and concentration dependent manner that was in consistent with other study reported that cytotoxic effects of chitosan were negatively correlated with its molecular weight ((Maeda et al, 2004 and Huang et al., 2004) used as nanoparticles but in the present study, we used beetles derived chitosan against A549 and HCT-116 cancer cell lines. MTT assay was used to determine the viability and cytotoxic effect on cell lines, but in other studies of Senthil Kumar Kuppusamy and Jayaprakash Karuppaiah (2002), recording that chitosan was added as 62.5 µg/ml and after 48 hours, showed 52.7% viability which is lower than the previous results. This showed a great interference between chitosan and colorectal cancer cell lines, while in other studies performed by Huang and Khor recorded that the chitosan IC50 value was less that than determined in the present study that may be attributed of the source of chitosan and % of acetylation. Also, they recorded that the Mw of chitosan was not related to its toxicity and proved that their derived chitosan IC50 value was of a narrow range recording 1.1 to 1.2 mg/ml using MTT assay (Huang, Khor, & Lim, 2004). Anticancer potential of chitosan was noticed and Our data was in agreement with another study evaluated the molecular mechanisms causing the cell cycle arrest and related genetic profile concerning cdk-1, cyclin A, cyclin B1, p21Waf1, and p27Kip1 and detected their protein using western blot analysis post different colorectal cell lines (HCT-115 / HCT-122 and Caco2), reporting a marked time-dependent decrease in cyclin A and B1 expression when cells were treated with 100 µM celecoxib. Cdk-1 protein levels also decreased, though to be for a lesser extent than those of both cyclins. Conversely, the expression of p21Waf1 and p27Kip1 increased in a time-dependent manner with a maximum at 24 h treatment. This effect occurred in all three cell lines irrespective of the COX-2 status. In contrast to celecoxib, SC560 cells did not change the expression of the cell cycle regulatory proteins tested.(Grösch, Tegeder, Niederberger, Bräutigam, & Geisslinger, 2001). Also, Our present study agreed with Salehi and behboudi study as they proposed that chitosan promotes ROS-mediated apoptosis and S phase cell cycle arrest in triple-negative breast cancer cells: evidence for intercalative interaction with genomic DNA (Salehi & Behboudi, 2017), that agreed with our present study about beetles' chitosan promotes apoptosis and S phase cell cycle arrest in lung and colorectal cancer cells anti-bacterial assay had been performed as some bacterial strains from gram-positive (pyogen and aureas) bacteria and gram-negative bacteria (salmonella-typhimurium). The suitability of chitosan as a food preservative is dependent on the kind of chitosan used and the matrix in which it is dissolved. Therefore, it is important in experiments with chitosan to use a well-characterized product of polymerization and acetylation degree. In this experiment, shrimp derived chitosan unlike the recent studies used beetles' derived chitosan. All the experiments in the other studies used chitosan, of a low polymerization degree (43kDa) and high deacetylation degree (94%) combination that seemed to be very favorable for the anti-microbial Balicka-Ramisz et al (2005) and Chien et al,2016). This can be derived from the relatively low values in comparison with other studies. On the contrary, Gram-negative bacteria seemed to be very sensitive for the applied chitosan while the sensitivity of the gram-positive bacteria varied greatly. The MIC for lactic acid bacteria was higher than 0.05% (w/v) while other Gram-positive bacteria were already inhibited at 0.006% (w/v). The MICs reported in the literature for specific target organisms (No et al, 2002). Some studies showed that pseudomonas sp. is the most strains resistant to chitosan (Rhoades and Roller, 2000; Jeon et al.,2001 and Rabea et al,2003). This is not inconsistent with our results and with the studies of (Alharbie and Ziad,2014 and Chien et al,2016). It seems in those studies that Gram-negative bacteria have great respond to chitosan unlike our result that show great

effect on gram-positive and low effect on gram-negative bacteria with usage of Beetles'-derived-chitosan. It seems that most published work was contrary to ours that gram-positive had less effect about used chitosan but with beetles' chitosan it seems it have a different result. Some studies showed that Gram-negative bacteria had more efficiency by using chitosan as nanoparticles, which disagree our results that gram-positive had greatly affected by beetles' chitosan than gram-negative. Gram-negative bacteria were more effective to chitosan nanoparticles than gram-positive bacteria.(Hassan, Mohamed, & Taher, 2016) . Qi et al. (2004); Balicka-Ramisz et al (2005); Tayel et al.(2010a); Islam et al. (2011); Ben-habiles et al. (2012); Rodrigues- Núñez et al. (2012) and Younes et al. (2014) recorded the antibacterial activities of chitosan against Salmonella sp., these records were not hassling with the activity of derived beetles' chitosan against *salmonella-typhimurium* obtained in the present study.

CONCLUSION

In this work, our finds indicate that beetles derived chitosan showed anticancer potential proved via evaluation of cytotoxicity that was concentration and cell type dependent, also, toxicity proved incidence of apoptosis that monitored via genetic profile assessment. Also, apoptotic profile was accompanied with high level of Early and late apoptosis. Also, derived chitosan showed a more pronounced antibacterial activity against gram-positive bacteria than gram-negative one.

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