



EGYPTIAN ACADEMIC JOURNAL OF  
**BIOLOGICAL SCIENCES**  
**ENTOMOLOGY**

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ISSN  
1687-8809

[WWW.EAJBS.EG.NET](http://WWW.EAJBS.EG.NET)

**Vol. 14 No. 2 (2021)**



**Preparation, Characterization and Anticancer Activity of Chitosan Prepared from the American Cockroach, *Periplaneta americana***

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**ARTICLE INFO**

**Article History**

Received:29/3/2021

Accepted:2/6/2021

**Keywords:**

Chitosan;  
Anticancer; Liver cancer; Breast cancer; American cockroach.

**ABSTRACT**

The exoskeleton of a cockroach plays a major role in protecting it from pathogenic microbes. It is well known that the main component of the exoskeleton is the chitin which is considered the source of chitosan. This study aimed at investigating the anticancer activity of chitosan prepared from the American cockroach. Chitosan was prepared and characterized with solubility in 1% acetic acid and Fourier transform infrared spectroscopy (FT-IR) analysis. The cytotoxicity of chitosan was investigated against Hepatoblastoma (HepG2) and Breast cancer (MCF7) cell lines by the MTT assay. The cytotoxicity has a positive relationship with the chitosan concentration. The half-maximal growth inhibitory concentrations (IC<sub>50</sub>) were 329 and 195 µg/ml with HepG2 and MCF7, respectively. Therefore, Chitosan may be a potential natural compound for the treatment of human hepatoblastoma (HepG2) and breast cancer (MCF7).

**INTRODUCTION**

Cancer is one of the major causes of mortality for humans globally, and the most frequent cancer forms in humans are lung, breast, prostate, and liver cancers. Breast cancer is the most commonly occurring cancer in women and the second most common cancer overall. While Liver cancer is the fifth most commonly occurring cancer in men and the ninth most commonly occurring cancer in women.

Many chemically manufactured anticancer medicines have caused harm to patients, mostly through immune system suppression. So, a lot of research has gone into finding and developing novel medicines based on natural products (Rayan, 2017; Yao *et al.*, 2017; Wright, 2017).

Natural products are considered as valuable substances that play an important role in resisting various kinds of diseases because of their safety, widely and diversity. Their availability in different kinds of insect within their various environments, biodiversity and adaptability make them the most successful group of all animals. Many major human diseases are transmitted by insects, which serve as vectors of diseases, but not get affected or infected with different pathogens, which proves that insects have a potent immune

system (Ashfaq *et al.*, 2018).

Globally, the second most important natural biopolymer is chitin that is a source of chitosan. Chitosan is an aminated polysaccharide abundant in nature, its chemical characteristics provide that chitosan a unique set of functional properties. The main characteristics of chitosan are its degree of acetylation and molecular weight, these have a determining effect on the chitosan functional properties, from its solubility and materials-forming capacity to biodegradability and diverse bioactive properties (Da-Silva *et al.*, 2021).

Some of the more notable recent discoveries in bioengineering natural products from insects with application or prospective utility in modern medicine are presented. (Ratcliffe, *et al.*, 2011). In this overview, the present research aimed at extracting and preparing chitosan with characterization from the adult cockroach, *Periplaneta americana* to explore its role as an anticancer agent against two human cancer cell lines: Hepatoblastoma cells (HepG2) and Breast cancer (MCF7).

## MATERIALS AND METHODS

### Insect Collection:

Adult cockroaches were collected by insect's net according to the method of Rentz (2014). Samples were classified according to key constructed by Mukha, *et al.* (2002).

### Preparation and Characterization of Chitosan:

#### 1. Preparation of Chitosan:

Chitosan powder was prepared according to the methods of Oduor-Odote *et al.* (2005), with some modifications as follows:

Adult cockroaches were killed by CO<sub>2</sub> gas. Subsequently, bodies were anatomized to extract the exoskeletons and then chopped into pieces. They washed with distilled water (DW) and dried at 50° C. Then it was homogenized, sieved, and weighted. Exoskeleton powder (250 g) was demineralized by stirring in 500 mL of 1N HCl for 2 h at 75 ° C. Then filtrated, and washed with distilled water until become neutral, and finally washed with absolute ethanol then dried in the oven at 50° C for 12 h and weighed. The demineralized powder was stirred in sodium hydroxide 2.5 % (w/v) by magnetic stirrer for 5 h at 100° C with ratio (1 g: 20 ml), then filtrated and washed with distilled water until become neutral and finally washed with absolute ethanol then dried in the oven at 50° C for 12 h and weighed (in this phase, the deproteinization of the sample has occurred). The deproteinization step was repeated twice. After the deproteinization, the sample was incubated in chloroform: methanol: distilled water solution (1:2:4) for discoloration and bleaching treatment for 1 h. After that 21 g of sample were left in 2 liters of sodium hydroxide 50 % (w/v) overnight, and then stirred by magnetic stirrer for a total of 8h at 120°C, then filtrated, washed with distilled water and absolute ethanol then drying in the oven at 50°C for 12h (in this phase, the deacetylation of the sample occurred).

#### 2.Characterization of Chitosan:

Chitosan tested for solubility in acetic acid 1 % (v/v) with a ratio of 1 g: 100 ml. The chemical structure of chitosan was confirmed by FT-IR analysis in wave range 4000–400 cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup> at Micro Analytical Center, Faculty of Science, Cairo University. The degree of deacetylation of chitosan was calculated by equation (1) according to (Baxter *et al.* 1992):

$$DDA\% = 100 - \left[ \frac{A_{1655}}{A_{3450}} \times 115 \right] \dots\dots\dots (1)$$

Where: A<sub>1655</sub> and A<sub>3450</sub> are absorptions of bands at 1655 and 3450 cm<sup>-1</sup>, respectively.

### 3. Anticancer Activity of Chitosan:

#### 1. Cell Lines:

Hepatoblastoma cells (HepG2), and Breast cancer (MCF7) were supplied by Tissue Culture Department, VACSERA, Egypt. HepG2 and MCF7 cells were cultured in 75 cm<sup>3</sup> tissue culture flasks [Griner- Germany] using RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin. The cells were grown in a humidified atmosphere (incubator) of 5 % CO<sub>2</sub> at 37 °C.

#### 2. Cytotoxicity of Chitosan:

Cytotoxicity of chitosan on viability and proliferation of HepG2 and MCF7 cell lines was estimated by the MTT assay (Bahuguna *et al.*, 2017). The cells were seeded at  $2 \times 10^4$  cells/well in 100 µL of complete culture medium in 96-well tissue culture plates (Nunc-Denmark) and cultured for 24 h at 37 °C. The culture medium is discarded and replaced with fresh media only in control wells, Chitosan was added to the cells at concentrations of 1000, 500, 250, 125, 63, 16, 8, 4, 2, 1, 0.5 and 0.3 µg/ml and incubated in 5 % CO<sub>2</sub> at 37 °C for 24 h. After incubation, MTT solution (0.5 mg/ml, 50 µl) was added to each well and the cells were incubated for a further 4 h. The supernatant was then carefully removed, and 50 µL of DMSO was added to each well. The optical density (OD) was measured using ELX-800 Biotek- USA ELISA reader. The viability percent was calculated using the formula:  $\text{Viability \%} = (\text{OD of treated cells} / \text{OD of control cells}) \times 100$ . The half-maximal growth inhibitory concentrations (IC<sub>50</sub> values) were calculated by fitting the survival curve using MasterPlex 2010 software (MiraiBio, Hitachi Solutions America, Ltd). All examinations were done in triplicates and the listed data are the average of the obtained results.

## RESULTS

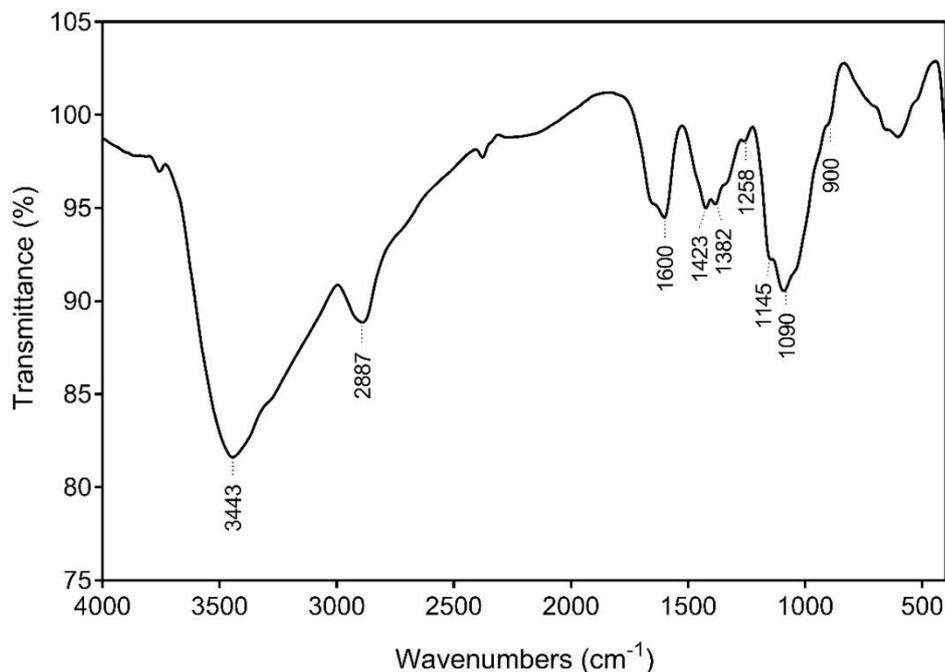
### 1-preparation and Characterization of chitosan:

Chitosan was extracted and prepared from *Periplaneta americana* (Blattodea: Blattidae) and characterized with solubility and FT-IR. For Solubility, 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 % as showed in figure (1).



**Fig.1:** Chitosan powder soluble in 1 % acetic acid with ratio 1 g / 100 ml.

While the FT-IR analysis, the main functional groups of chitosan structure were detected (Fig 2 and table 1). The absence of band absorption at 1540 cm<sup>-1</sup> indicated successful deproteinization for chitin. The high intensity of the 1600 cm<sup>-1</sup> band and the absence of absorption band around 1650 cm<sup>-1</sup> were pronounced to the presence of the NH group with a successful deacetylation process of chitin. The degree of deacetylation (DDA) was 72.8 %.



**Fig.2:** FT-IR spectrum of chitosan prepared from the American cockroach

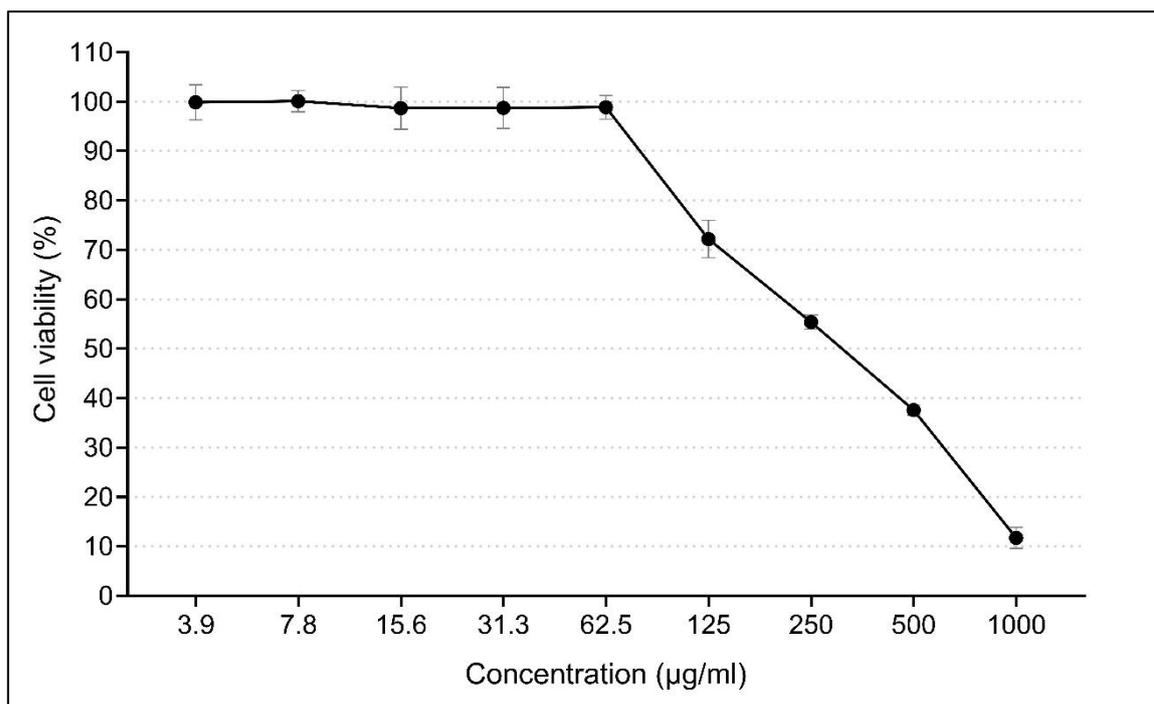
**Table (1):** Characteristics of Fourier transform infrared (FT-IR) spectral data for the American cockroach chitosan.

Peak Wavenumber (cm <sup>-1</sup> )	Function group
3433	$\nu$ NH and $\nu$ OH Free
2887	$\nu_{as}$ CH (-CH <sub>2</sub> )
1600	$\delta$ NH (R-NH <sub>2</sub> )
1423	$\delta_{as}$ CH (-CH <sub>2</sub> )
1382	$\delta_s$ CH (-CH <sub>2</sub> )
1258	$\nu$ CN or $\delta$ OH
1145	$\nu_s$ (C-O-C)
1090	$\nu$ (C <sub>3</sub> -O) OH group
900	Vibration of Pyranose ring skeletal

Where:  $\nu$ : Stretching,  $\nu_s$ : Symmetric Stretching,  $\nu_{as}$ : Asymmetric stretching,  $\delta$ : Bending,  $\delta_s$ : Symmetric bending and  $\delta_{as}$ : Asymmetric bending.

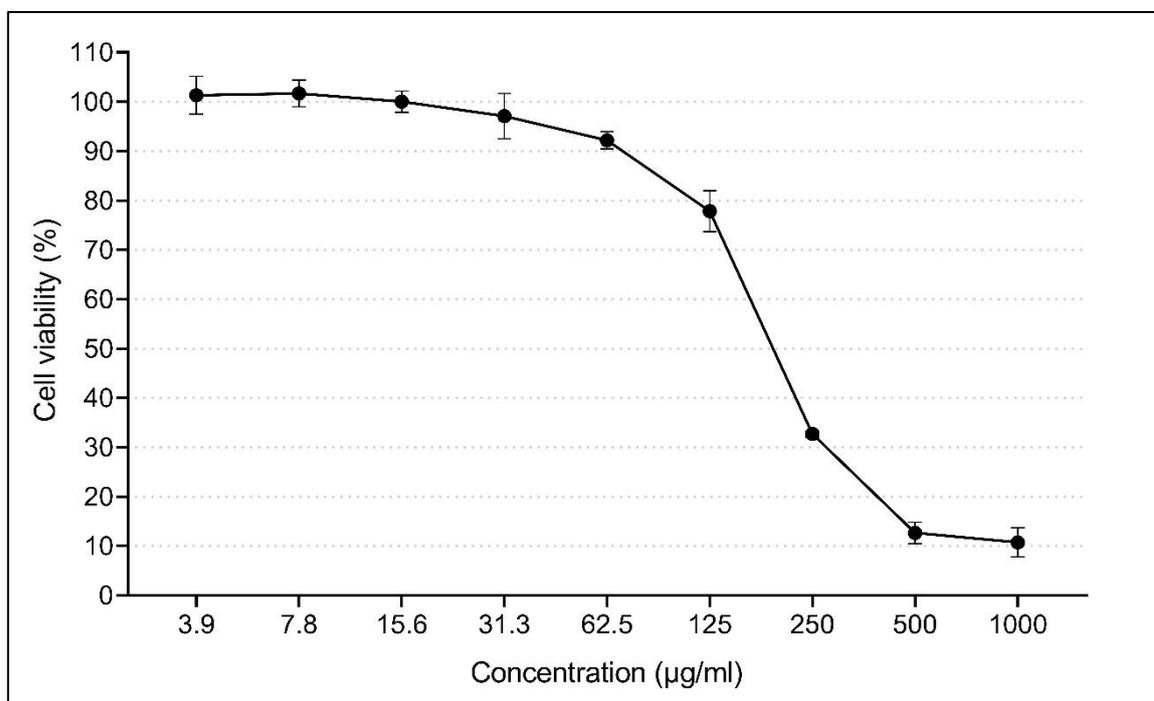
## 2-Anticancer Activity of Chitosan:

Effects of chitosan on the viability of HepG2 and MCF7 cell lines using MTT assay were investigated. Chitosan reduces the cell viability of HepG2 and MCF7 cell lines, where the cell viability was inversely proportional to the concentration of chitosan. On one hand, the chitosan doesn't show cytotoxicity on HepG2 at concentrations of 3.9, 7.8, 15.6, 31.3 and 62.5  $\mu\text{g/ml}$ , while it showed 27.8, 44.6, 62.5 and 88.3 % cytotoxicity at a concentration of 125, 250, 500 and 1000  $\mu\text{g/ml}$ , respectively (Fig. 3). The half-maximal growth inhibitory concentration (IC<sub>50</sub>) was 329  $\mu\text{g/ml}$ .



**Fig.3:** Cytotoxicity of chitosan on (HepG2) cell line.

On the other hand, showed 7.8, 22.1, 67.3, 87.3 and 89.2 % cytotoxicity on MCF7 at concentrations of 62.5, 125, 250, 500 and 1000 µg/ml, respectively (Fig. 4). The half-maximal growth inhibitory concentration ( $IC_{50}$ ) was 195 µg/ml.



**Fig.4:** Cytotoxicity of chitosan on (MCF7) cell line.

## DISCUSSION

From the results of this study, chitosan was completely dissolved in 1 % acetic acid in agreement with Peter (1995), the solubility of chitosan in acetic acid 1 % was

indicated that chitosan with the degree of deacetylation above 50 %. Fourier transform infrared spectroscopy (FT-IR): In agreement with Basseri *et al.* (2019) and Hahn *et al.* (2020), the broad peak around  $3430\text{ cm}^{-1}$  was represented the stretching vibration of NH and OH groups in chitosan. While the asymmetric stretching vibration of CH (-CH<sub>2</sub>) was observed at a band around  $2887\text{ cm}^{-1}$ , this result was approximate with the results of Rady *et al.* (2018) and Abuelmakarem *et al.* (2019). The detection of the bending vibration for NH (R-NH<sub>2</sub>) at a band around  $1600\text{ cm}^{-1}$  was identical with reports of Hassan *et al.* (2016), Mohan *et al.* (2019) and Lahouti and Naeimi (2020).

The bending vibration of CH (-CH<sub>2</sub>) was represented at bands around  $1423\text{ cm}^{-1}$  and  $1382\text{ cm}^{-1}$ , this result was hassling with the results of Divya *et al.* (2017) and Ibitoye *et al.* (2018). In agreement with Zvezdova (2010) and Subhapradha *et al.* (2013), the Stretching of the C–O–C has appeared around band  $1145\text{ cm}^{-1}$ . By looking at the present result and that of Zhang *et al.* (2011) and Chae *et al.* (2018), the stretching of hydroxyl groups of C-OH was observed at bands around  $1090\text{ cm}^{-1}$  and  $1042\text{ cm}^{-1}$ . While the stretching of pyranose skeletal ring was seen at band  $900\text{ cm}^{-1}$ , this result was similar to the results of Karimi *et al.* (2013) and Wanule *et al.* (2014).

The antitumor effect of chitosan has been studied towards the same carcinoma cells by Shen *et al.* (2009) and the chitosan demonstrated the protective effect on the proliferation of Hepatoblastoma (HepG2) cells and suppress tumor growth. Also, Azuma *et al.* (2014) considered chitosan as a promising factor for the development of an antitumor agent against hepatocellular carcinoma cells (HepG2). Salehi *et al.* (2017) reported the antitumor properties of chitosan and its inhibitory effect on the proliferation of three kinds of breast cancer cell lines (MCF7), (MDA-MB-231) and (T47D). Similar to the results of Taher *et al.* (2019), the anti-proliferative effect of chitosan was recorded against human breast cancer cell lines.

In addition, chitosan can be considered as an effectual anticancer agent, in light of this, the potential cytotoxic effect of chitosan was investigated against human bladder cell carcinoma (RT112) by Younes *et al.* (2014), and the authors detected that chitosan showed an important reduction in cell number, where it able to induce the cytotoxicity with the high IC<sub>50</sub>. Their results are in accordance with our findings as shown. Also, Chakraborty *et al.* (2012) reported that modified chitosan had in vitro cytotoxicity against HeLa cell lines in mice. While Hosseinzadeh *et al.* (2012) proved that chitosan inhibited the viability of colon carcinoma cell line (HT-29).

Ganesan *et al.* (2020) reported that chitosan had a cytotoxic effect on Human larynx carcinoma (Hep2) cells and Human embryo rhabdomyosarcoma (Rd) cells, similar to Abuelmakarem *et al.* (2019), Chitosan-tripolyphosphate showed increased cytotoxicity and decreased cell viability against colon cancer cells (Caco2). Generally, chitosan and its derivatives are considered as a potential naturally anti-cancer polysaccharide (Qi *et al.*, 2005 and Xu *et al.*, 2009).

## CONCLUSION

From the study, chitosan was prepared successfully from the American cockroach with a degree of deacetylation (DDA) was 72.8 %. Also, the main functional groups of chitosan were confirmed by FT-IR. MTT assay showed a significant inhibitory effect of chitosan on the proliferation of Hepatoblastoma cells (HepG2) and Breast cancer (MCF7) in vitro, and the IC<sub>50</sub> was 329 and 195  $\mu\text{g/ml}$  for HepG2 and MCF7, respectively. Breast and liver cancers are among the most common types of cancer that are regarded as one of the main causes of mortality in humans worldwide. Chitosan can be a potential natural compound for the treatment of human hepatoblastoma (HepG2) and breast cancer (MCF7).

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