

## Extraction & characterization of Chitosan from Nile water crawfish *Procambarus clarkii*, Egypt

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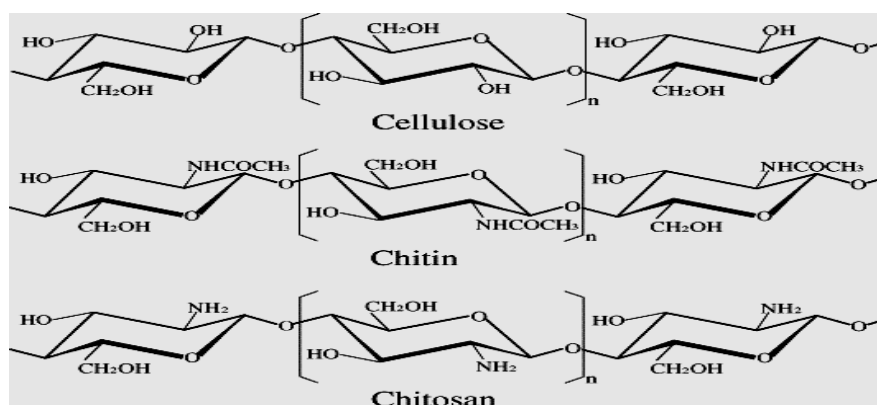
### Abstract

Chitin and its derivatives, particularly chitosan, have been recently used in many life science applications, and many studies are daily conducted to reach chitosan optimum characteristics which enable many applications to be done in order to serve the need of the society. The aim of this study is to extract chitin from a source which is locally abundant and with low society utilization. Chitin was extracted from freshwater crawfish *Procambarus clarkii* which is abundant in Egypt River Nile. The extraction was performed by three different protocols, the first method (DPMCA) was done by deproteinization, demineralization, and decolorization then to obtain the chitosan; deacetylation was done, the second method (DMPCA) was achieved by reversing the deproteinization with the demineralization process and finally a method described by YU Xin-xin in 2013. It was observed that DPMCA extraction method is the best for chitin extraction from *Procambarus clarkii* as it gave the maximum chitin yield (28%) and subsequently the maximum chitosan yield (25.8%) compared with the other tested methods and those described in the literature (Vanitha *et al.*, 2018). It shows a degree of deacetylation = 96.27%, Nitrogen content = 6.73%, Hydrogen content= 6.57%, Carbon content= 35.04%, Sulphur content= 0% , Moisture content= 6.6% and the Molecular weight = 7.138 Da (g/mol) which is considered as low molecular weight. All these characteristics help in using the extracted chitosan in many biomedical applications.

**Keywords:** Crawfish, *Procambarus clarkii*, Egypt, extraction, characterization, chitosan.

## Introduction

As global awareness of the chitosan importance increases day by day, new approaches were developed in order to enhance its applications. Chitosan is a linear polymer of a ( $\beta$ 1-4)-linked 2-amino-2-deoxy-b-D-glucopyranose and is easily derived by *N*-deacetylation of chitin. It is a heteropolymer of fibre-like substance consists of *N*-acetyl glucosamine and *D*-glucosamine units that are distributed among the chain. Chitin comes after cellulose as the second most abundant organic compound. The shellfish waste of *crustaceans* such as crawfish, shrimps and crabs is considered the main source of chitin (Vanitha *et al.*, 2018).. Now crawfish *Procambarus clarkii* is considered to be one of the biggest crustaceans in the freshwater ecosystems (Fisher, 2006).



**Figure 1: Structure of Cellulose, Chitin, and Chitosan**

The acetyl content of the polymer is considered as the actual difference between chitosan and chitin as shown in Figure 1 (Vanitha *etal.*, 2018).. Chitosan has chemical properties as it is a highly basic polysaccharide, linear polyamine, has reactive amino groups, reactive hydroxyl groups and chelates many transitional metal ions. Chitosan has also some biological properties as it is a biopolymer that shows biocompatibility and biodegradability which are affected by the degree of deacetylation and molecular weight. Other important characteristics of chitosan are viscosity and solubility. Viscosity is important in determining the molecular weight of chitosan, as higher chitosan molecular weight often renders higher viscous solution which is not desirable for handling; it was found that viscosity related to particle size such that the smaller particle size gives higher viscosity than the larger one.

Chitosan is characterized by its higher solubility in diluted organic acids such as acetic acid, formic acid and lactic acid than inorganic acids; also the solubility of chitosan is poor in solvents with PH 7.00 (Vanitha *etal.*, 2018).. Biocompatibility, biodegradability and non-toxicity of chitosan enable it to be used in biomedical, industrial, environmental and biotechnological investigations (Pradip Kumar Dutta, 2004). One of the examples of using chitosan in such applications was done as they coated the titanium with Biodegradable chitosan nanoparticle coatings and used it as a drug carrier for enhancing the delivery of bone morphogenetic protein (BMP-2) to promote osteoblasts proliferation, differentiation and attachment (Poth N. *etal.*, 2015) Azam Aliasghari (2016) studied the effect of nano-chitosan as antimicrobial agent against carcinogenic streptococci .On the other hand chitosan can also be used in environmental applications; it is used as an adsorbent to treat rice mill wastewater by high removal efficiency of pollutants. (as chemical oxygen demand (COD) and total suspended solids (TSS) ) and protection of ecosystem by preventing the production of secondary pollutants (Thirugnanasambandham *etal.*, 2013). In addition, Chitosan is now used for promising biotechnological

applications such as chitosan-dextran sulfate nano-capsule which is used for delivery of dsRNA into *Penaeus monodon* (*P.monodon*) post larvae to silence the Monodon baculovirus (MBV) structural gene of p74 (Ramesh kumar, 2016).

Chitosan is also used to form matrix for enzyme immobilization as chitosan cryogel beads were used to immobilize glucose oxidase for glucose biosensor fabrication (Amin Fatoni, 2016) as well as it is used in the structure of scaffolds used in tissue engineering (No and Meyers, 1989). This study aims to Recycle the exoskeleton of Nile water crawfish *Procambarus clarkii* as an abundant source for chitosan extraction, using three different protocols and finding out the best method of extraction that provides the highest yield and best chitosan characterizations.

## Materials and Methods

### 1. Collection of the samples and processing

The crawfish *Procambarus Clarkii* was collected by fishermen from the Nile River. The exoskeletons of crawfish were separated from the muscles and viscera then washed by tap water. Exoskeletons were boiled for 15 min on the heater, dried and incubated in an oven at 70 °C for 3 hr; the samples were left to dry in room temperature then grinded by grindery. Samples were kept in transparent Nylon bags with silica preserving sacks and kept away from light.

### 2. Extraction of chitin

**The powder was treated by three different methods:**

#### 2.1 DPMCA Chitin Extraction method:

The processed powder was deproteinized by 3.5% (w/v) NaOH solution with 10 ml/gm solvent to solid ratio, for 3 hr at 65°C with constant stirring. Then the sample washed with water until clarity of solution. The deproteinized sample was splitted into two samples where the first one was demineralized by 1N HCl at

room temperature and constant stirring for 60 min (coded by Sample 1) and the second one for 30 min (coded by Sample 2), with 10 ml/gm solvent to solid ratio. After filtration of the samples from HCl, they were washed with tap water until clarity of solution then left for 3 hr to dry in the oven.

The extracted chitin was decolorized with acetone for 10 min and dried for 3 hr at 30 °C then bleached with 0.315 % (v/v) sodium hypochlorite (NaOCl) solution (containing 5.25% available chlorine) for 5 min at ambient temperature. Chitin was washed and left to dry for 2 hr until complete dryness and crispness at 65 °C.

### **2.2 DMPCA Chitin Extraction method:**

The Second method is DMPCA which used to be the most common method in chitin extraction in the literature where some described it as the standard procedure (Marei NH, 2016 ). In this method, the demineralization step achieved first then deproteinization (J. Majtán, 2007) (Entsar S. Abdou, 2007).

### **2.3 (YU Xin-xin, 2013) Chitin Extraction method:**

Finally, a method published in 2013 (YU Xin-xin, 2013) which proceeds in the same sequence of DMPCA but the decoloration step was achieved by using 5 gm/ L potassium permanganate for 1 hr and 10 gm/ L oxalic acid for 20 min with 10 ml/gm solvent to solid ratio instead of acetone and sodium hypochlorite.

## **3. Production of Chitosan**

Chitosan was obtained from deacetylation of chitin. The chitin samples were soaked in 50% sodium hydroxide solution (NaOH) for 24 hr, then autoclaved for 1 hr and washed till neutrality with tap water. The solubility of the samples was tested after they have been dried to ensure the conversion from chitin to chitosan. This was accomplished by dissolving 0.1gm of each sample into 1% and 5% acetic acid.

#### 4. Characterization of Chitosan:

##### 4.1 Viscosity and Molecular weight

The viscosity was measured for the soluble sample (DPMCA) by using Ostwald capillary viscometer. The relative viscosity was determined for chitosan polymer dissolved in 5% acetic acid and 1M KCl to obtain the intrinsic viscosity which is useful in calculating Molecular weight. Chitosan viscosity was measured for different concentrations (0.1%, 0.2%, 0.3%, 0.4 %, 0.5 %), to establish a standard curve then calculate the intrinsic viscosity.

The molecular weight is calculated by the following equation:  $(\eta) = KM^a$ . Where (K) and (a) are empirical viscometric constants at which  $K= 1.81 \times 10^{-3}$  ml/gm and  $a= 0.93$  (Entsar, 2007).

##### 4.2 Elemental analysis

The sample was measured for its N<sub>2</sub> (nitrogen), H<sub>2</sub> (hydrogen), C (carbon), S (sulphur) and Moisture content by vairo El III-Elementar machine (Germany) in the micro-analytical centre, Cairo University, Egypt.

##### 4.3 Thermal Gravimetric analysis (TGA)

The sample was measured for its thermal stability in Housing & Building national research center, Doki, Egypt using Shimadzu TGA-50H instrument under nitrogen atmosphere with heating rate 10 °C/ min (Entsar, 2007).

##### 4.4 Moisture content:

3 gm of the sample was placed in a vacuum oven at 125 °C and the decrease in the weight was measured, which corresponds to the loss of water molecules as shown in the following equation:

$$\text{Moisture content \%} = (W_1 - W_2) / W_1 \times 100$$

$W_1$  and  $W_2$  are the weights of wet and oven dried samples, respectively (Fernandez-Kim, 2004).

#### 4.5 Degree of deacetylation

Samples were calculated for its degree of deacetylation by the following equation:

$$\frac{6.857 - C\%}{1.7143} / N\%$$

Where C is the Carbon content and N is the Nitrogen content which is obtained from the elemental analysis test 6.857 and 1.7143 respectively

#### 4.6 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of the commercial chitosan and extracted chitosan were achieved by FTIR Jasco 4100 (Japan) in the micro-analytical centre, Cairo University, Egypt.

#### 4.7 X-ray Diffraction (XRD)

The sample was measured for its crystallinity in Housing & Building national research center, Doki, Egypt.

## Results and Discussion

### 1. Chitin yield and solubility

The yield of the extracted chitin as a dry weight over the total weight of processed crawfish shell by performing different extraction methods is shown in Table 1.

**Table 1: Yield of extracted chitin by different protocols**

Type of treatment	DPMCA (S <sup>1</sup> )	DPMCA (S <sup>2</sup> )	DMPCA	(YU Xin-xin, 2013)
Chitin yield %	21.4%	28.5%	11.6%	9.7%

The solubility of the samples in 1 % and 5 % acetic acid for 24 hr is clear in

Table 2.

**Table 2: solubility test for the extracted chitosan**

Type of treatment	Solubility in 1 % acetic acid	Solubility in 5 % acetic acid
1- DPMCA (S <sup>1</sup> )	Partially soluble	Partially soluble
2- DPMCA (S <sup>2</sup> )	Partially soluble	Soluble
3- DMPCA	Insoluble	Insoluble
4- (YU Xin-xin, 2013)	Insoluble	Insoluble



It is obvious that the samples treated with DPMCA method show the highest chitin yield. Furthermore, S<sup>2</sup> of DPMCA gives 25.8 % of chitosan which is soluble in 5% acetic acid this is higher than the yield obtained in the studies of (Marei, 2016 ), (Al-Manhel, 2016), (Vanitha *etal.*, 2018).& (Iqbal, 2014). The chitosan yield is slightly lower than that of chitin due to the loss of the mass during deacetylation step(Vanitha *etal.*, 2018).. The improper solubility of the rest of the samples may be due to improper deproteinization, As a result, the remaining proteins or amino acids interfere with the solubility of chitosan(Vanitha *etal.*, 2018).. Consequently, further characterizations were done for the dissolved sample of chitosan which is DPMCA (S<sup>2</sup>).

## 2. Viscosity and Molecular weight of extracted chitosan :

The molecular weight was calculated for DPMCA (S<sup>2</sup>) to be 7.138 Da; using the intrinsic viscosity. (Vanitha *etal.*, 2018).reported that the molecular weight of chitosan samples ranged from 674.49 to 10,596.62 Da (Vanitha *etal.*, 2018). implies the extracted chitosan in this study have relatively, low molecular weight compared to the literature. This enhances its ability to be used in medical applications (Struszczyk, 2006). Some of the factors that affect chitosan viscosity are; ionic strength, temperature, Molecular weight, degree of deacetylation, pH and bleaching. As the two samples (DMPCA and YU Xin-xin method) appear almost white while the DPMCA samples are coloured, this could be a possible explanation of the poor solubility and viscosity as cited in (Moorjani, 1975).

### 3. Elemental analysis and Moisture content of extracted chitosan:

**Table 3. N2 content, H2 content, C content, S content and Moisture content**

Sample treatment	Nitrogen content	Hydrogen content	Carbon content	Sulphur content	Moisture content
DPMCA (Sample 2)	6.73%	6.57%	35.04%	Nil	6.6%

According to Struszczyk which reported that chitosan has an average moisture content from 5% to 15 % which is compatible to that obtained in this study (Struszczyk, 2006). Also, Iqbal et al stated that the moisture content of chitosan extracted from shrimp is about 8.5 % when it was treated using the same conditions in this study (Iqbal, 2014). These variations may be due to the source of chitin and drying conditions, also chitosan is hygroscopic in nature hence it can be affected by moisture absorption during storage (Khan, 2002). However, for medical applications chitosan moisture content ranges from (5% - 15%) (Struszczyk, 2006). Nitrogen content reported in previous studies was 8.03% for chitosan extracted from Louisiana crawfish using the same method of DPMCA (Vanitha *et al.*, 2018). which is higher than that obtained in this study (6.73%) and another studies (7.06% to 7.97%) which are conducted on crab and shrimp. The higher nitrogen content is an indication of the presence of protein residues which implies improper deproteinization process (Rutherford & Austin, 1978). However, Nitrogen and carbon contents are useful measurements in order to measure chitosan degree of deacetylation.

### 4. Degree of deacetylation

The degree of deacetylation is an important parameter which reflects the chitosan quality and how the step of chitin conversion to chitosan was a successful process. According to the elemental analysis the degree of deacetylation was measured for

(DPMCA)  $S^2$  to be 96.27 % this is close to the range (56% -99% with an average of 80%), whereas it is higher when compared to the values (68% & 75.3%) obtained from another study conducted on crawfish but using different analytical method (Vanitha *et al.*, 2018).. The degree of deacetylation depends on the source of purification, type of analytical methods employed, sample preparation, and type of instrument used (Khan, 2002).

## 5. Thermal Gravimetric analysis (TGA)

TGA curves of chitosan are used to measure the thermal stability of chitosan (Figure 2). Two endothermic peaks are observed. The first peak appears at 77.9 °C corresponds to the loss of water. The second one emerges at 298.68 °C, which is quite close to what was described in literature (Pereira, 2013) (Entsar, 2007).

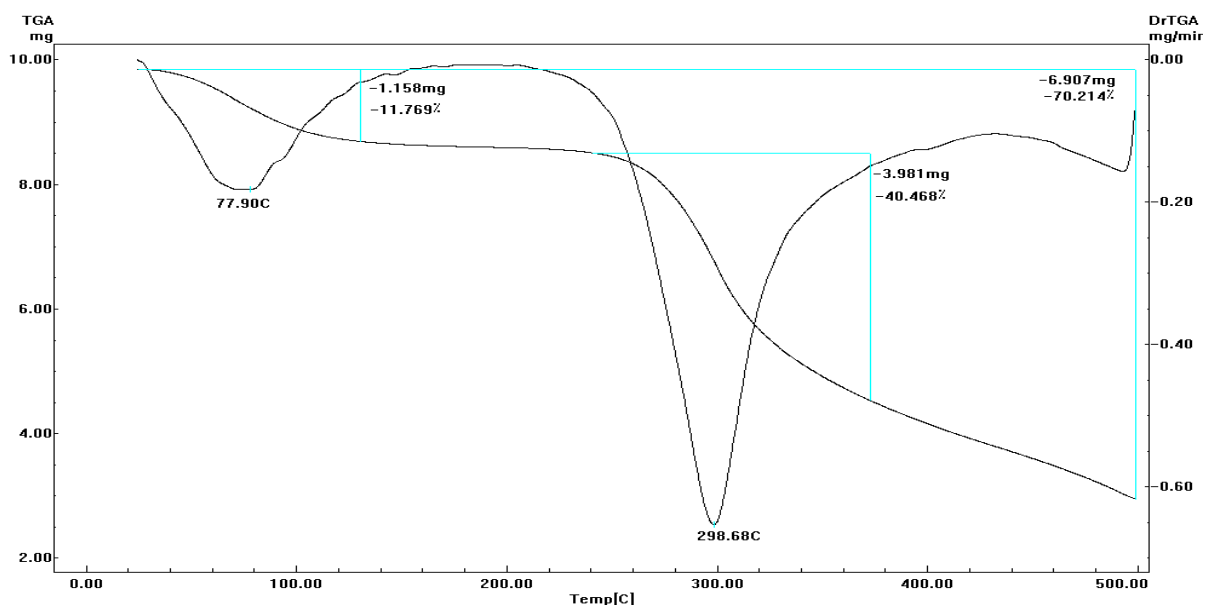


Figure 2: TGA of the extracted chitosan

## 6. Fourier transform infrared spectroscopy (FTIR)

To confirm the structure of chitosan FTIR analysis was performed for the sample extracted by DPMCA S<sup>2</sup> method and a commercial chitosan. **Error! Reference source not found.**<sup>3</sup> and **Error! Reference source not found.**<sup>4</sup> represent the IR spectra of extracted and commercial chitosan respectively. The IR spectra of both extracted and commercial chitosan seem to be similar; generally the bands appear nearly in (3000-3500, 1400-1650, 2885, 1650, 1589, 1326 and 1080 cm<sup>-1</sup>) are corresponding to (NH<sub>2</sub> and OH, C=O, C-H, Amide I, Amide II, Amide III and C-O-C) respectively, these bands are characterizing chitosan structure (Marei, 2016).

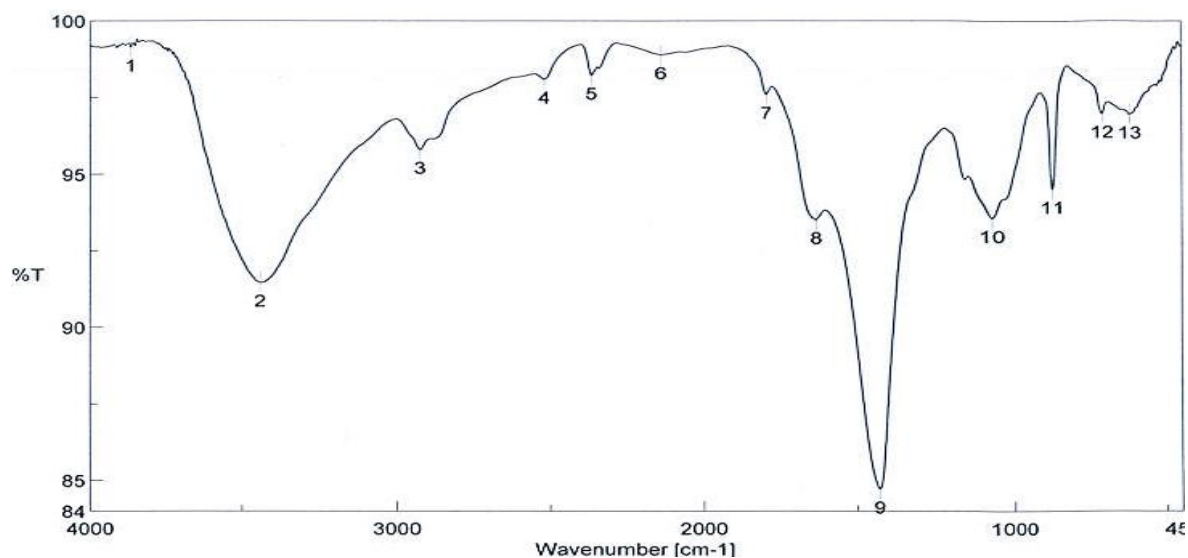
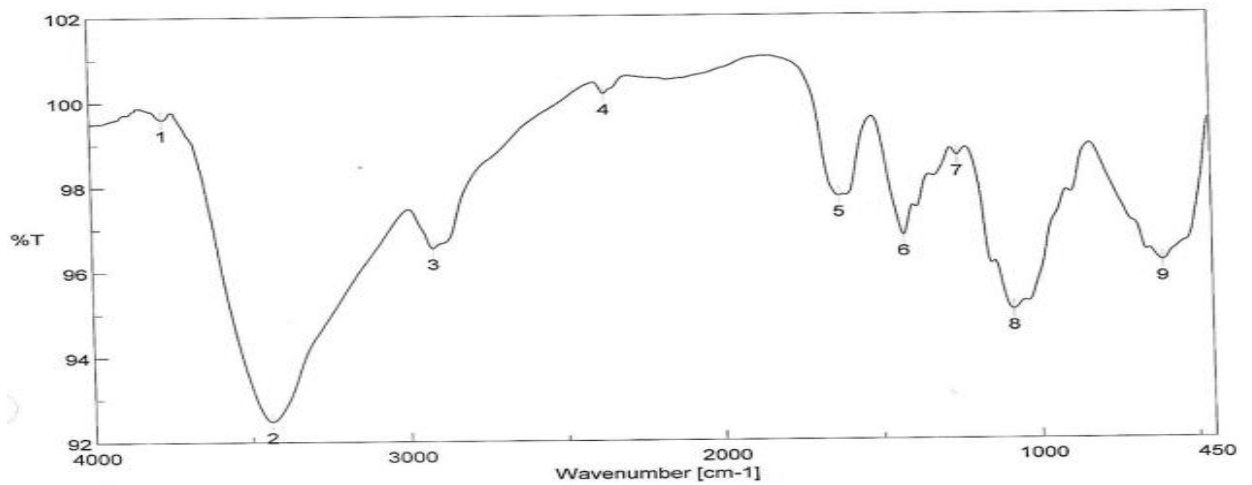


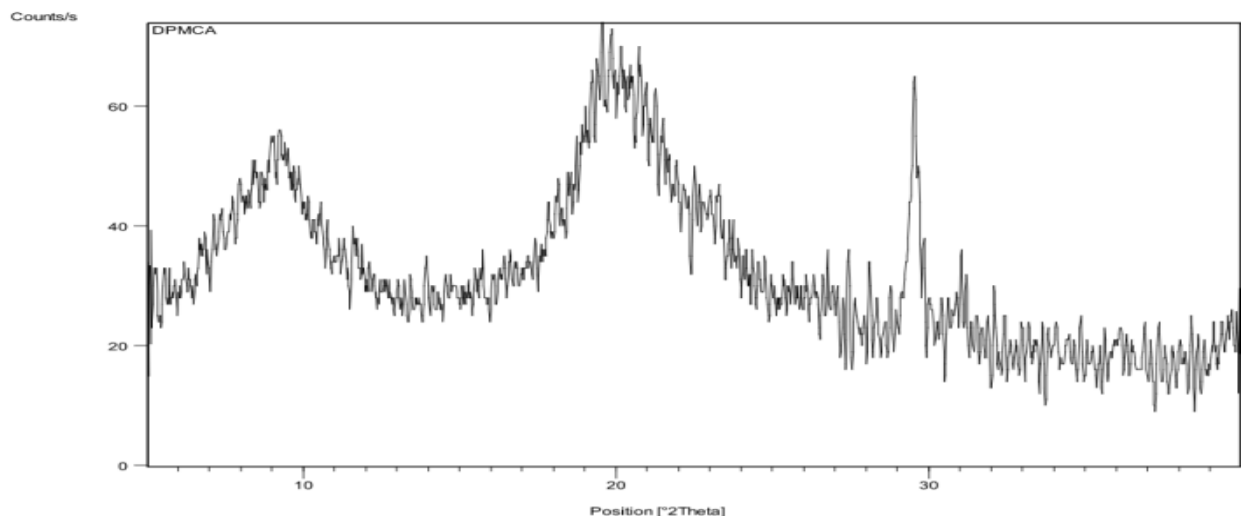
Figure 3: IR spectrum of commercial chitosan



**Figure 4: IR spectrum of the commercial chitosan**

### 7. X-ray Diffraction (XRD)

There are two peaks in  $10.67^\circ$  and  $19.99^\circ$  those are close to the peaks in  $10^\circ$  and  $20^\circ$  which are characterizing to chitosan which appear in Figure 5. and correspond to the (020) and (110) planes of the crystalline lattice (Marei, 2016)



**Figure 5: X-ray diffraction of the extracted chitosan**

## Conclusion

This study aimed to determine the best method of chitin extraction and chitosan production by chitin deacetylation from crawfish *Procambarus clarkii* shell. The fact that *Procambarus clarkii* is naturally and locally abundant organism with low cost encourage its utilization in producing an eco-friendly and multi-advantage product. The characteristics of the extracted product were measured to test the product quality. It was found that the valid method among the tested methods is DPMCA (Deproteinization, Demineralization, Decolorization and Deacetylation) as it gives the best chitin & chitosan yield which is 28 % & 25% respectively. Chitosan degree of deacetylation is about 96.27%. It has a low molecular weight of 177.42 Da (gm/mol). This study provides a method for obtaining chitosan with high yield and good characterizations from a locally abundant source which may facilitate its utilization in medical applications besides its biocompatible and biodegradable properties.

## Acknowledgement

This experimentation was accomplished by the aid of biotechnology/biomolecular chemistry program coordinators, prof.Dr. Entsar S. Abdou, Youssef Wahba, Ahmed H.M. Elwahy and Dr. Haiman.

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