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Chemical characterization, biocidal and molluscicidal activities of chitosan extracted from the crawfish *Procambarus clarkii* (Crustacea: Cambaridae)

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ABSTRACT

Invertebrates have many useful pharmacological activities. The present investigation aims to study some of these activities in the red swamp crawfish *Procambrus clarkii* against a varying number of pathogens. Results showed that raw chitosan had molluscicidal and larvicidal effects on both *Biomphalaria alexandrina* snailsand*Schistosoma mansoni* larvae. Also, the current study confirmed the inhibitory action of chitosan against three gram-positive bacteria *(Streptococcus faecalis, Bacillus subtilis*, and *Staphylococcus aureus*) and three gram-negative bacteria (*Neisseria gonorrhoeae*, *Pseudomonas aeuroginosa* and *Escherichia coli*). Additionally, in the current study chitosan revealed antifungal activity against *Candida albicans* but no effect was detected against *Aspergillus flavus*. The present results indicated that chitosan can be used as a molluscicidal agent, a rodenticide against the albino rat and insecticide against adults of the tiny black ants, *Monomorium minimum*. For the first time, under field conditions, raw chitosan has herbicidal activity against *Cyperus rotundus* weeds of chitosan were demonstrated. Conclusively, these natural by-products can be used as biocidal agents against different pathogens.

INTRODUCTION

Chitosan is derived from chitin that found in arthropod exoskeletons (Kim, 2018). It had many special biological, chemical and physical properties (Aam *et al*., 2010). The antimicrobial resistance is one of the major health problems that encountered the world and this focused the light on developing newer antimicrobial therapies that diminishes this resistance (Arias and Murray, 2009).

Schistosomiasis is endemic in tropical region and it affects billions of people worldwide (Rees *et al*., 2019). It is caused by trematode worms of the genus *Schistosoma* which need the presence of an intermediate host to transmit (Feitosa *et al*., 2018). *Biomphalaria* genus is the intermediate hosts for *Schistosoma mansoni* (Ibrahim and Sayed, 2019). To control the spread of this disease, several strategies have been used

through controlling the intermediate host (Omobhude *et al*., 2017) either by using biological materials or chemical synthetic materials (Ibrahim, 2018). Recent researches search for an ideal alternative instead of the chemical molluscicides as these chemicals pollute the water ecosystem and have high costs (Ibrahim and Ghoname, 2018).

Rodents and insects caused inconvenience to humans, livestock, and can transmit zoonotic disease (Meerburg *et al*., 2009). These Pathogens might include parasites (e.g., toxoplasmosis), viruses or bacteria (Meerburg *et al*., 2009). It was assumed that the cost associated with the transmission of rodent-borne diseases is similar to the losses due to insects and rodents in plant production (Bordes *et al*., 2015). To decreases these losses, chemical insecticides and rodenticides were used but the latter polluted water, soil and air. Human also, is in risk of the danger of these chemicals, as the residues of the insecticides are detected in his body (Ansari *et al*., 2013). Therefore, biopesticides might be used as bio- alternative tools to eradicate insect and rodent pests with safe activity to the environment and human.

Consequently, weeds caused a lot of damage to crops and lead to low income, where it shared in the uptake of nutrients and water with the cultivated plants (Sopeña *et al*., 2009). Chemical herbicides caused serious environmental problems in natural water, soil, and foodstuffs (Itodo *et al*., 2017). A material to be herbicide might be partially or totally control or kill plants by absorption then lead to the death of the plant (Itodo, 2019). The purpose of this study was to screen for natural by-products which contain active compounds that could be used as biocides. This objective was specifically achieved by studying the bactericidal, molluscicidal, insecticidal, rodenticidal and herbicidal activities of raw chitosan extracted from the red swamp crawfish, *Procambrus clarkii*.

MATERIALS AND METHODS

1. Raw material preparation:

Procambarus clarkii (Crustacea: Cambaridae) were collected from the River Nile at Giza Governorate, Egypt. They were packed in plastic bags and transported to the Laboratory of Invertebrates, Zoology Department, Faculty of Science, Cairo University. The exoskeleton of the crawfish *Procambarus clarkii* was dissected and washed, then dried for 6 hours at 60 °C in an oven. The product blended with an electric blender to get the fine powder that passes through 300 µ sieve (Takiguchi, 1991; El-Naggar *et al*., 2018; Hamdi, 2019).

1.1. Extraction of chitosan:

Exoskeleton of crawfish *procambarus clarkii*; (carapace, chelipeds, legs, telson, tergum and sternum of abdomen) was dissected and scraped to discard the sticky tissue, washed and dried for 6 hours at 60 °C in an oven. The product was crushed with a mortar and blended with an electric blender to get crawfish exoskeleton powder that passes through 300 µ sieve.

Four subsequent separately steps; deproteinization, demineralization, decolorization, and deacetylation process were carried out to obtain chitosan powder from crawfish using the methodology described by (No and Meyers, 1989; Hadi, 2013; El-Naggar *et al*. 2020).

1.2. Chemical characterization of chitosan:

a- IR characterization of chitosan:

The infrared spectra were recorded in potassium bromide disks on a pyeUnicam SP-3-300 and Shimadzu FT IR 8101 PC infrared spectrophotometers.

b- UV-visible spectra measurement:

UV-visible spectra were recorded using a Shimadzu UV-visible1800 spectrophotometer. Sample was analyzed in triplicate at 25 ˚C at a scattering angle of 90 ˚C.

2. Snails:

Biomphalaria alexandrina snails (9- 10 mm) in length provided from Medical Malacology Laboratory, Theodor Bilharz Research Institute (TBRI), Giza, Egypt were kept in plastic aquaria (16 x 23 x 9 cm). The aquaria were provided with dechlorinated aerated tap water (10 snails/ L) and oven dried lettuce leaves for feeding.

3. Cercariae and miracidia:

Both were obtained from Malacology lab, Theodor Bilharz Research Institute (TBRI).

4- Bioassay tests:

4.1. Molluscicidal screening:

To calculate LC_{50} and LC_{90}, five serial dilutions were prepared (80, 70, 60, 55, and 50 mg/l) from the stock solution from chitosan on the basis of W/V using dechlorinated tap water. Ten snails were incubated for each concentration with three replicates (WHO, 1983). Also, three replicates of the control group of the same size were dipped only in dechlorinated water. The exposure period was 24 h then, the recovery for 24 h in dechlorinated tap water. The median lethal concentration was calculated by probit analysis.

4.2. Miracidicidal and cercaricidal activities:

One hundered *S. mansoni* miracidia and 100 *S. mansoni* cercariae in 5ml of water were mixed with 5ml of LC_{50} of chitosan. Another 10 ml of dechlorinated tap water containing either 100 miracidia or 100 cercariae were kept as control (Ritchie *et al*., 1974, Abdel-Ghaffar *et al*., 2016). The experiment was observed under a dissecting microscope to show the changes in the movement. Stationary larvae were considered dead and then mortality rate is calculated (Obare, 2016).

5. Antimicrobial activity (Sensitivity tests):

This experiment was done using a modified Kirby-Bauer disc diffusion method (Bauer *et al*., 1966). Where, 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media to get the count of approximately 10^8 cells/ml for bacteria or 10^5 cells/ml for fungi (Pfaller *et al*., 1988). 100 µl of microbial suspension was spread onto agar plates. Plates were inoculated with filamentous fungi *as Aspergillus flavus* at 25^{o}C for 48 hrs; Gram (+)

bacteria as *Streptococcus faecalis, Staphylococcus aureus, Bacillus subtilis*; Gram (-) bacteria as *Escherichia coli, Pseudomonas aeuroginosa* and *Neisseria gonorrhoeae.* they were incubated at 35-37^{o}C for 24-48 hours and yeast as *Candida albicans* incubated at 30^{o}C for 24-48 hours and, then the diameters of the inhibition zones were measured (Bauer *et al*., 1966). Standard discs of Ampicillin (Antibacterial agent), Amphotericin B (Antifungal agent) served as positive controls for antimicrobial activity but filter discs impregnated with 10 µl of solvent (distilled water, chloroform, DMSO) were used as a negative control. Blank paper disks (Schleicher and Schuell, Spain) with a diameter of 8.0 mm were impregnated 10µ of tested concentration of the stock solutions. A filter paper disc impregnated with a tested chemical was placed on the agar which then was diffused and placed the chemical around the disc. The area of no growth around the disc is known as a "zone of inhibition". For the disc diffusion, the zone diameters were measured with slipping calipers of the National Committee for Clinical Laboratory Standards. Agar-based methods were simpler and faster than broth-based methods (Matar *et al*., 2003).

6. Insecticidal activity

Groups of 50 adults of the tiny black ants *Monomorium minimum* (Insecta: Formicidae) were reared in petri-dishes (10 cm in diameter and 15 mm depth) containing 10 gm sugar which was covered by a fine mesh cloth for ventilation. The cultures were maintained in a growth chamber set at 30°C. Adult insect's utilized for toxicity test. A concentration of 10 mg/l was prepared from chitosan based on W/V using dechlorinated tap water placed in a bottle and sprayed, while the extracts were replaced by distilled water in the control. The treatments were held in the laboratory under ambient conditions ranged between 25-28^{o}C. The experiment is replicated three times and percentage of mortality was calculated.

7. Herbicidal activity:

Cyperus rotundus weeds were subjected to 62.5 mg/l of chitosan solution and the results were recorded daily to show the changes in the green color of the weed till it became faint yellow.

8. Rodenticidal activity

To know the experimental rodenticidal effects of raw chitosan on wistar rats, the animals were randomly divided into three groups of seven each weighing 120 to 125 g. Chitosan was suspended in 1 % glacial acetic acid and fed by intragastric tube daily for a week.

Group 1: normal control (1ml of 1 % glacial acetic acid).

Group 2: 1 ml of chitosan (100mg/kg in 1% glacial acetic acid).

Group 3: 1 ml of chitosan (30mg/kg in 1% glacial acetic acid).

9. Statistical analysis:

The median lethal concentration (LC_{50}) was calculated for the 24h tests. Toxicity results were analyzed by Probit analysis (Finney, 1971) and were reported as a concentration resulting in the death of 50 % of the test organisms after 24h. The obtained results were analyzed by one way ANOVA using SPSS v. 15.0.

RESULTS

Results:

1. IR characterization of chitosan isolated from the crawfish *P. clarkii*

Infrared spectrum of chitosan showed characteristic vibrational bands due to its unique chemical skeleton with multiple functional groups **(Fig. 1)**. The spectrum showed main peaks owing to stretching vibrations of hydroxyl groups (OH) in the range from 3850 cm^{-1} to 3443 cm^{-1}, which are overlapped to the stretching vibration of N-H; and C–H bond in methylene (–CH_2; υ = 2922 cm^{-1}) and methyl (–CH_3; υ = 2853 cm^{-1}) groups, respectively. Additionally, bending vibrations of –CH_2 and –CH_3 functional groups were also detected at υ = 1370 cm^{-1} and υ = 1417 cm^{-1}, respectively. Moreover, absorption in the range of 1644–1556 cm^{-1} was associated to the vibrations of carbonyl groups (C=O) of the amide group CONHR (secondary amide, υ= 1644 cm^{-1}) and to the vibrations of protonated amine group (δ_{NH3}, υ= 1556 cm^{-1}). Also, absorption in the range from 1156 cm^{-1} to 1021 cm^{-1} has been attributed to vibrations of carbonyl (CO) group. The band detected at υ= 1094 cm^{-1} is associated to asymmetric vibrations of carbonyl (CO) in the oxygen bridge resulting from deacetylation of chitosan. The bands in the range 1094–1021 cm^{-1} are assigned to υ_{CO} of the ring COH, COC and CH_2OH. The minor band at 869 cm^{-1} links to wagging of the saccharide skeleton of chitosan.

2. UV-Visible spectra

The UV spectrum of chitosan was carried out at (25± 2) with 1nm resolution, the analysis revealed that the wavelength of chitosan is 336 nm.

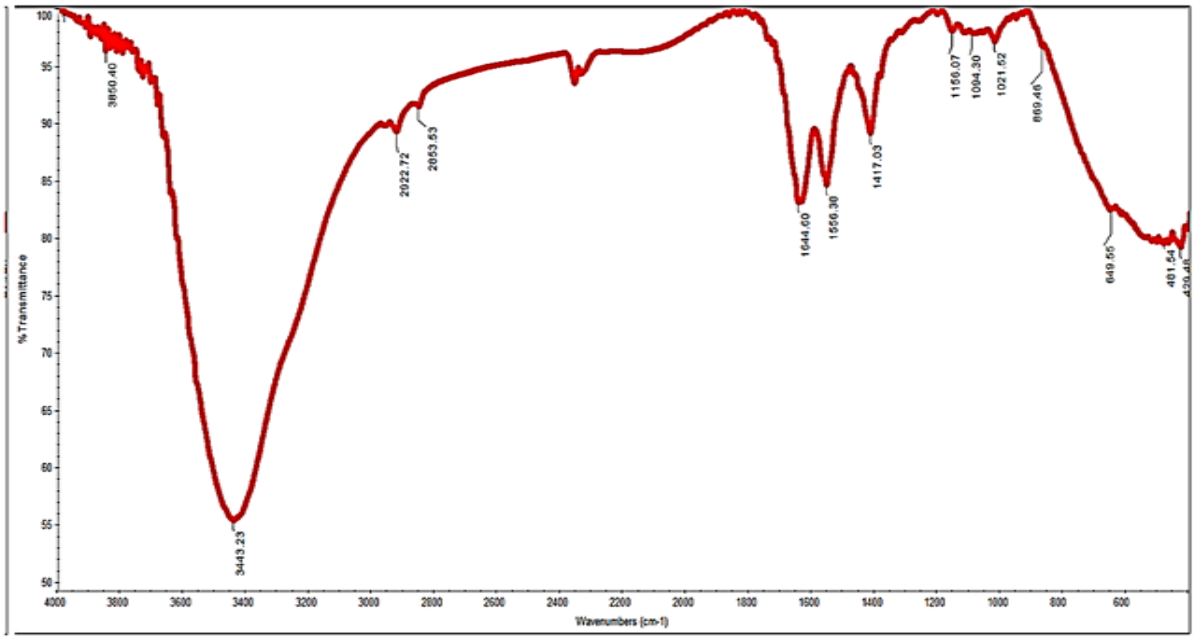

Fig. 1. Infrared spectrum of the chitosan (KBr)

3. The molluscicidal activity of chitosan

Exposure of adults of *B. alexandrina* snails after 24h followed by 24 h for recovery caused a half lethal mortality at LC_{50} 62.5 mg/l (Table 1and Fig.2).

Table 1. Molluscicidal activity of chitosan against adult *B. alexandrina* snails after 24h of exposure followed by 24 h for recovery, done by Probit analysis

Snails	LC_{10} (mg/l)	LC_{25} (mg/l)	LC_{50} (mg/l)	Confidence limits of LC_{50} (mg/l)	LC_{90} (mg/l)	Slope
B. alexandrina	53.2	57.6	62.5	57.1- 70.4	71.8	1.1

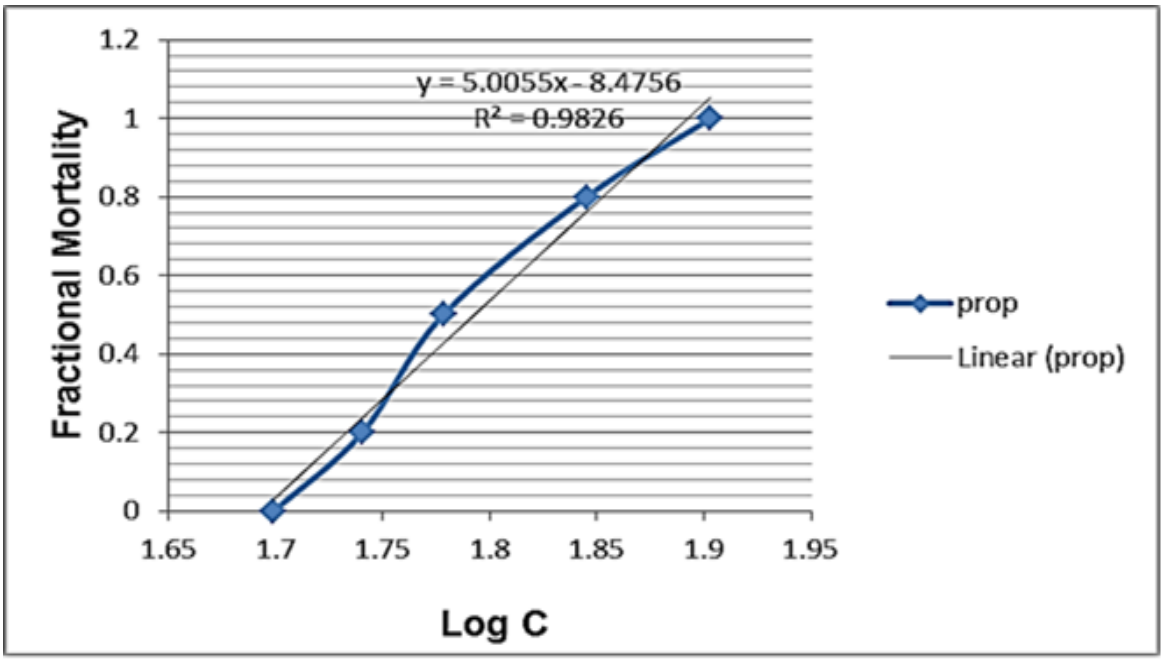

Fig.2. Graph shows the molluscicidal activity of chitosan against adult *B. alexandrina* snails after 24 h of exposure followed by another 24 h for recovery

4. Miracidicidal and cercaricidal activities:

The present results showed that *S. mansoni* miracidae exposed to LC_{50} of chitosan had 50% mortality after 10 min. All miracidia died after 20 min compared to 5% death for the control group (Table 2). Also, the cercariae exposed to LC_{50} had 50% mortality after 10 min, and that all cercariae died after 25 min compared to 15% for the control group.

Table 2. Effect of LC_{50} of chitosan on *Schistosoma mansoni* miracidia and cercariae

		% cumulative mortality of miracidia and cercariae after the following intervals (min)					
Concentration ((ppm)		5	10	15	20	25	30
Miracidia	Control	0	0	0	5	10	15
	Treated	25	60	90	100		
Cercariae	Control	0	0	0	0	15	20
	Treated	20	50	70	90	100	

5. Antimicrobial activity:

The antibacterial activity of chitosan reported probable effect against three gram positive bacteria *Streptococcus faecalis, Staphylococcus aureus, Bacillus subtilis* and three gram negative bacteria *Neisseria gonorrhoeae, Escherichia coli and Pseudomonas aeuroginosa*. Additionally, in the present study chitosan revealed antifungal activity against *Candida albicans* but no effect against *Aspergillus flavus* as shown in Table 3.

Table 3 Bactericidal and fungicidal effect of chitosan

Sample		**Inhibition zone diameter (mm/ mg sample)**							
		Bacterial species						**Fungi**	
		G+			**G-**				
		B.subtilis	***S.aureus***	***S. faecalis***	***E. coli***	***N. gonorrhoeae***	*P. aeuroginosa*	***A. flavus* (Fungus)**	***C. albicans* (Fungus)**
Standard	Ampicillin Antibacterial agent	26	21	27	25	28	26	--	--
	Amphotericin B Antifungal agent	--	--	--	--	--	--	17	21
Control: DMSO		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Treated: Raw chitosan		29	29	30	30	29	29	0.0	29

4. Insecticidal activity:

The % of mortality of adults *Monomorium minimum* exposed to chitosan as treated and water as control group was recorded at different time intervals till the end of the experiment along with the control group (Table 4 and Fig.3). The mortality was carefully checked and seriously decreased with the increase in incubation period when compared to the control. The control ants group was active till 12 h and then a slight decrease in their active state was observed.

Table 4 Insecticidal effect of chitosan on adults *Monomorium minimum*

Concentration (ppm)		% cumulative mortality of adults *Monomorium minimum* after the following intervals (mins)					
		30	60	90	120	150	180
M. minimum	Control	0	5	10	10	15	20
	Treated	25	30	50	60	90	100

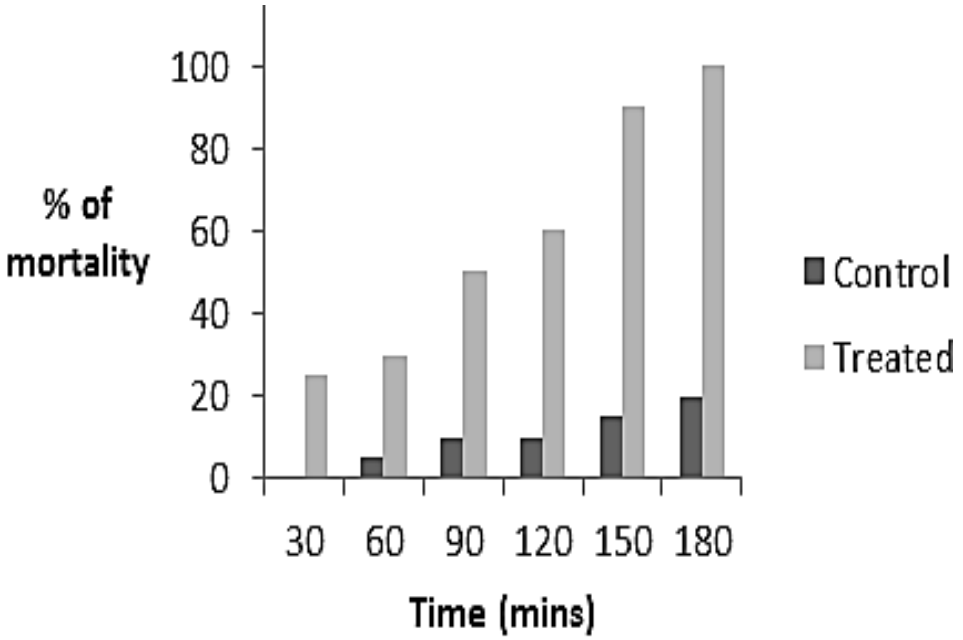

Fig. 3. Histogram shows the percentage of mortality of *M. minimum* exposed to chitosan (treated group) and water (control group)

5. Herbicidal activity:

The present results showed that chitosan had a herbicidal activity against *Cyperus rotundus* weeds, where, it caused death of these weeds after 10 days of exposure to 62.5 mg/l (Fig.4).

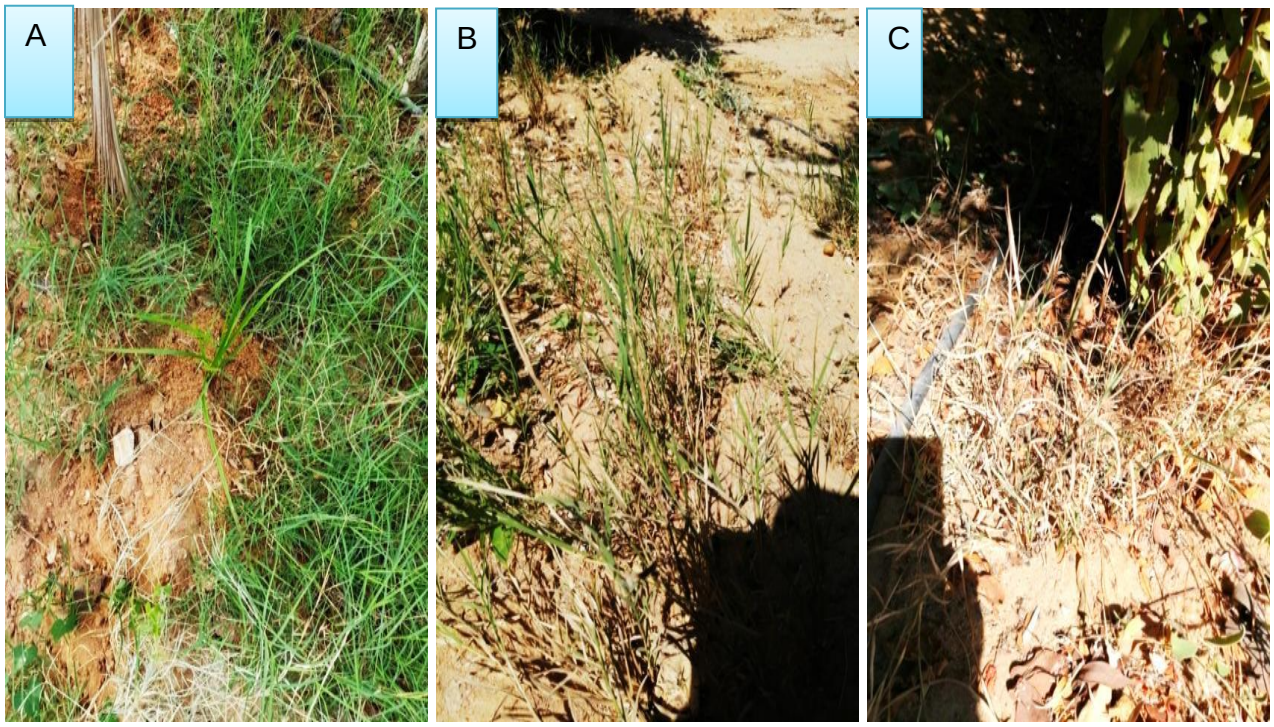

Fig.4. Photographs show the herbicidal activity of chitosan on *Cyperus rotundus* weeds after exposure to 62.5 mg/l. A) After 3 days of exposure, B) after 5 days, and C) after 10 days.

6. Rodenticidal activity:

No mortality or abnormal behavioral or physiological changes was observed among the control group or those treated with low dose. Wistar rats that consumed high doses of chitosan exhibited poisoning symptoms with swollen stomach within the first day and 86-100% died between 5 to 6 days after treatment as shown in Table 5.

Table 5 Rodenticidal effect of raw chitosan against the wistar rat for 7 successive days

Time to death (days)	Concentration (mg/kg)		
	Group 1 1ml of 1 % glacial acetic acid	Group 2 100 mg/kg in 1% glacial acetic acid	Group 3 30 mg/kg in 1% glacial acetic acid
	% of mortality		
1	0	15	0
2	0	42	0
3	0	57	0
4	0	71	0
5	0	86	0
6	0	100	15
7	0	100	15

DISCUSSION

The spectrum showed main peaks owing to stretching vibrations of hydroxyl groups (OH) in the range from 3850 cm^{-1} to 3443 cm^{-1}, which are overlapped to the stretching vibration of N-H; and C–H bond in methylene ($–CH_2$; $\upsilon = 2922\ cm^{-1}$) and methyl ($–CH_3$; $\upsilon = 2853\ cm^{-1}$) groups, respectively. Additionally, bending vibrations of $–CH_2$ and $–CH_3$ functional groups were also detected at $\upsilon = 1370\ cm^{-1}$ and $\upsilon = 1417\ cm^{-1}$, respectively. Moreover, absorption in the range of 1644–1556 cm^{-1} was associated to the vibrations of carbonyl groups (C=O) of the amide group CONHR (secondary amide, $\upsilon = 1644\ cm^{-1}$) and to the vibrations of protonated amine group (δ_{NH3}, $\upsilon = 1556\ cm^{-1}$). Furthermore, absorption in the range from 1156 cm^{-1} to 1021 cm^{-1} has been attributed to vibrations of carbonyl (CO) group. The band detected at $\upsilon = 1094\ cm^{-1}$ is associated to asymmetric vibrations of carbonyl (CO) in the oxygen bridge resulting from deacetylation of chitosan. The bands in the range 1094–1021 cm^{-1} are assigned to υ_{CO} of the ring COH, COC and CH_2OH. The minor band at 869 cm^{-1} links to wagging of the saccharide skeleton of chitosan (Negrea *et al*., 2015). Moreover, the UV spectrum of chitosan sample was matched with the previous reports (Rabea *et al*., 2005; Negrea *et al*., 2015; Vijayalekshmi, 2015). Additionally, the most important concept is the obvious variation between chitosan samples according their physiochemical characteristics like degree of deacetylation, crystallinity, and molecular weight (Cheung *et al*., 2015). Another, vital issue is the little solubility of chitosan in most polar and non-polar solvents as well as

neutral and alkaline media which limits its biomedical application, and also its chemical characterization especially when the analysis is based on complete solubility in specific solvents like deuterated solvents of NMR and UV analyses (Negrea *et al*., 2015).

Significant pests include bacteria, arthropods, molluscs and vertebrates, especially rodents, cause problems to the biodiversity that require management (Hansen *et al*., 2016). Recently, researches focused on studying the antimicrobial activity of chitin and chitosan against different microorganisms, such as bacteria and fungi (Limam *et al*., 2011). Chitosan in the present study has been investigated as an antimicrobial agent against some gram-positive and gram-negative bacteria. Moreover, chitosan has been reported to have fungicidal activity. Goy *et al*. (2009) found that chitosan was more effective against gram-positive bacteria (e.g. *Bacillus megaterium, Staphylococcus aureus, Lactobacillus plantarum, etc.)* than for gram-negative bacteria *(e.g. E. coli, Pseudomonas fluorescens, Salmonella typhymurium,,* etc.). These results are in good accordance with the present results on both bacteria and fungi.

Regarding the molluscicidal activity of chitosan against *B. alexandrina* snails, the half lethal concentration was LC_{50} 62.5 mg/l. According to (WHO, 1993), the half lethal concentration (LC_{50}) of a molluscicidal material should not exceed 100 mg\l. Therefore, the present results verified molluscicidal activity of the raw chitosan. These results coincide with (Khidr, 2018) who stated that both chitosan and nano-chitosan could be used as promising molluscicidal agents against the land snails *Eobania vermiculata* and *Monacha obstructa*.

Additionally, the present study showed that chitosan has a larvicidal effect on both *S. mansoni* miracidae and cercariae. Consequently, it will decrease the transmission of schistosomiasis and can be used in the control strategies of this disease. On the same line Oliveira *et al*.(2012) stated that chitosan play a vital role in increasing the immunity against the invading cercariae of *S. mansoni.*

The excessive uses of the synthetic pesticides caused the development of resistance in the treated animals (Ishaaya *et al*., 1995). In addition, it polluted the environment and caused many problems to the living creatures and this led the concern of both the scientists and the public in recent years to found natural products. These natural pesticides are considered as a new promising alternative for pest control as they reduce negative impacts on human health and the environment (Rehman *et al*., 2009). In the present investigation, the invertebrate extracts as bioactive chitosan was screened for its pesticidal effect against different pests, the tiny black ants *Monomorium minimum* and wistar rat.

Previous studies reported that the unmodified chitosan had low insecticidal activity against larvae of *S. littoralis*, but its activity was increased when it was chemically

modified (Rabea *et al*., 2005). This contradicts with the current results that record 95% mortality of *M. minimum* produced over by the end of the experiment detected within 3 hrs after exposure to chitosan spray.

The large scale and intensive use of chemical herbicides lead to more pollution in the ecosystem and transferred to the edible products. Researchers focused on how to overcome these harmful side effects of these chemical compounds to save soil and water, animal and human health (Marin-Morales *et al*., 2013). Glyphosate had more chronic side effects on environment (Battaglin *et al*., 2014), and this herbicide was reclassified by the World Health Organization as a carcinogenic material to humans in 2015 (Bai and Ogbourne, 2016). New concerns and strategies should be in consideration In order to search for safer, low cost, selective, and environmentally biodegradable herbicides. However, because of environmental concerns associated with the use of synthetic herbicides and emergence of numerous herbicide-resistant weed biotypes, substantial efforts have been made to design alternative environmental friendly weed-management strategies (Akbar *et al*., 2014). Natural compounds from plants can be used as model for herbicide production (Schabes and Sigstad, 2007). In the present investigation, raw chitosan extracted from the crustacean *Procambarus clarkii* revealed herbicidal activity for the first time and it expected to be of low or no hazard to the biodiversity.

Rattus spp. has harmful effects on the environment, crops and can transmit diseases (Meerburg *et al*., 2009). To decrease their populations many methods were used like physical tools, chemical rodenticides, biological/cultural methods (Witmer *et al*., 2012). The recent focus is on the natural rodenticide which is safe to environment with reduced reliance on chemicals (Oji *et al*., 1994). The obtained results revealed that raw chitosan has a rodenticidal effect.

CONCLUSION

In this study, the work was systematically scanned for the biological activities of crustacean by-products as raw chitosan against many pathogens. Based on the results of the current study, raw chitosan could serve as a source of biopesticides. Further studies are needed to isolated effective natural constituents from the animal extracts for the management of pathogens.

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