Research Article

Larvicidal Property of the Acidified Chitosan from Marine Crab Shell Wastes against *Aedes aegypti*

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Submission Date: 15-04-2020; Revision Date: 19-06-2020; Accepted Date: 22-06-2020

ABSTRACT

Dengue outbreaks cause elevated death toll yearly leading to human health concerns, particularly in tropical areas. The basic way to reduce mosquito densities is targeting its larval stage through alternative control management. This study investigates the use of chitosan, extracted from *R. ranina* crab shells obtained as common throwaways of seafood restaurants, as larvicide against *A. aegypti.* Powderized crab shells undergo through deproteinization and demineralization to make chitin and further converted into chitosan by deacetylation. Batches of 30 third instar larvae were used for each treatment prior for larvicidal bioassay. Mortality was recorded after 24 and 48 hr exposure. Data were pooled from all replicates for analyses. Regression analysis of log-probit was determined to calculate the lethal concentration (LC_{50}). Results revealed that acidified chitosan have increasing mortality rate of *A. aegypti* larvae as concentration increases. Among all treatments, 10000 ppm acidified chitosan possessed the highest toxicity effect against *A. aegypti* larvae and noticeably effective as the commercial larvicide. Acetic acid has no significant change in the mortality indicating that the larvicidal activity was due to the acidified chitosan. The LC_{50} was estimated at 6654.181 and 4942.489 ppm after 24 and 48 hr, respectively. With longer exposure, repressed growth and disintegration of body tissues of *A. aegypti* larvae were observed. This may suggest that the acidified chitosan has a potential to disrupt metabolic responses and inhibit growth development. This study emphasizes the utilization of waste materials can be developed as good alternative larvicides that are environmentally safe and inexpensive.

Key words: *Aedes aegypti*, Chitosan, Larvicidal, Lethal concentration, Mortality rate, Mosquitoes, *Ranina ranina*.

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INTRODUCTION

Mosquito vector of fatal diseases are considered important human health concern globally that brings high death rates every year, mostly in tropical countries.[1-3] In the Philippines, the predominant mosquito vector is *Aedes aegypti* that can transmit dengue fever and the more severe form, dengue hemorrhagic fever or infectious acute thrombocytopenic purpura.[4-6] A huge dengue outbreak in the country was reported by the Department of Health (DOH) during 2018. A total of 216,190 cases had been recorded from January 1 to December 31, 2018 and there were 1,083 deaths.[7] In the succeeding year, a national dengue epidemic was declared by the DOH in the rising wake of 146,062 cases with 622 deaths recorded since January 1 to July 20, 2019, 98% higher rate than the previous year.[8] The spread of dengue are complicated to foresee, but can be controlled through preventive ways.

One of the most effective way of reducing and controlling mosquito densities is by directly targeting its larval stage. Mosquito larvae are fragile and less scattered to which they are most vulnerable to mosquito

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DOI : 10.5530/ajbls.2020.9.22

control compounds.[9] The larva of *A. aegypti* has the ability to survive, develop and tolerate a wide range of water condition from pH level of 4 to 11.[10] Synthetic larvicides and insecticides have been traditionally used to control mosquitoes[11] but, their continuous application may lead to the development of genetic resistant mosquito vectors and also cause toxic effects to non-target organisms, including humans. It can cause itching, burning, numbness and behavioral problems.[12-14] The use of organic or natural product is an alternative way to inhibit the growth of mosquitoes[11,15,16] since it contain specific compounds that can be used as larvicide, adulticide and repellent against vectors that transmit diseases.[17]

Chitosan is a natural, biocompatible, biodegradable, non-carcinogenic, hypoallergenic and antibiosis straight-line polysaccharide composed of *N*-acetyl glucosamine and glucosamine units which is a deacetylated derivative of natural chitin.[18,19] Chitosan was determined to be soluble in acidic solutions.[20] Several studies reported that the application of chitosan can be used as a treatment for water pollution,[21] bacterial infection,[22] fungal infection,[20] inflammation,[23] cancer cells,[24] microbial infestation and wound healing.[25] Chitosan has been recognized to possess insecticidal effect against the lepidopterans such as the oleander aphid *Aphis nerii* and cotton leaf worm *Spodoptera littoralis*.[26] The antiviral property of the chitosan showed that it could be used as a substitute for chemical pesticide in controlling infection of vector insects.[19] Chitosan is mainly found on the exoskeleton of marine crustaceans such as shrimp, crabs and lobsters.[27,28] Marine crustaceans in southwestern Philippines are rich in abundance, particularly in Zamboanga City.[29] The most common throwaway shells from seafood restaurants in the locality are called "curacha" known as marine spanner crabs or red frog crabs (*Ranina ranina*) hence a good source of chitosan. This present study aims to utilize waste crab shells of *R. ranina* in obtaining acidified chitosan and tested against third instar *A. aegypti* larvae. Specifically, to determine which treatment will attain significant mortality rate and find out the lethal concentration (LC_{50}) exposed after 24 hr and 48 hr. Utilization of throwaways as organic material for vector control will not only benefit the environment but also the economy. It can be manufactured as an organic product in large amounts. This study may serve as a source of information for larvicidal product development based on crab shells.

MATERIALS AND METHODS

Collection of *A. aegypti* larvae

The method of using Ovicidal/Larvicidal (OL) trap was adapted to collect the eggs of *A. aegypti*.[30] It is a container filled with water and laid with a wood paddle as substrate. The OL traps were placed in shaded areas or placed in a typical breeding site to be oviposition by wild mosquitoes for several days. The life cycle of *A. aegypti*, when submerge in water, undergoes a complete metamorphosis with an egg, larva, pupa, and adult stage for about 8 to 12 days. A female mosquito can lay eggs in a range of 100 to 200 per batch. The eggs of *A. aegypti* can be distinguish by a long, black, ovoid-shaped and approximately 1 mm long. When eggs are visible on OL trap, the wood paddle is removed and then eggs are collected, dried and stored in vials at room temperature. The eggs of mosquitoes can survive in dry conditions for several months to one year and can be hatched in the presence of water.

Rearing of *A. aegypti* larvae

The wildly caught stored eggs were immersed in dechlorinated water to hatch simultaneously. The *A. aegypti* larvae were identified taxonomically and reared in the laboratory supplemented with flakes as food and kept at 25°C temperature with 12 hours dark-light photoperiod. Two-day old third instar larvae of *A. aegypti* were used as test organism prior for larvicidal bioassay.

Collection of Marine Crab Shells

Residual crab shells of *R. ranina* were collected as throwaways from different sea food restaurants in Zamboanga City, Philippines. The remaining viscera and tissues were carefully removed. The collected crab shells were washed repeatedly in a 500 ml beaker with deionized water and stirred continuously until it shows neutral pH level. It was then decanted and filtered in a muslin cloth. Crab shells were oven-dried at 60°C for 12 hr.[31]

Extraction of Chitosan

Using a mortar and pestle, 1000 g of oven-dried crab shells were pounded until powdery. The method of chitin extraction[32] was used with modifications. For deproteinization, the powdered crab shells was transferred in a 500 ml beaker with 4% NaOH[33] and heated at 60°C with constant stirring for one hour to

dissolve the proteins and sugars. It was then filtered with a strainer and the process was repeated until the filtrate becomes clear and colorless. For demineralization, 250 ml of 10% acetic acid[34] served as solvent and was slowly added to the solution with constant stirring at room temperature until no gas escaped. The filtrate was then washed with distilled water and filtered using ordinary filter paper until the liquid showed neutral pH level. It was oven-dried at 60°C for 12 hr. The dried deproteinized and demineralized sample obtained was chitin.

The chitin was further converted into chitosan by the process of deacetylation.[35] In a 500 ml beaker, 150 ml of 50% NaOH was added to chitin and then boiled at 100°C for 2 hr on a hot plate. The mixture was allowed to cool. The sample was washed continuously with hot distilled water at 60°C for three times and filtered in order to retain the solid matter product. The product obtained is chitosan showing whitish or pinkish form. The chitosan was kept and oven-dried at 60°C.

Test for the Presence of Chitosan

The ninhydrin test was used to detect ammonia or primary and secondary amines that determines the presence of chitosan. Usually when reacting with these free amines, a deep blue or purple color known as Riemann's purple is produced.[36] Chitosan is composed of *N*-acetyl glucosamine and glucosamine units.[37,38]

Determination of Larvicidal Property

Preparation of Treatments

A stock solution was prepared by weighing 0.90 g of chitosan and adding a 90 ml of 1% acetic acid which served as solvent. It was stirred and heated at 60°C. A 1% stock solution of acidified chitosan was produced. Following Borines *et al*.,[39] different concentrations of the stock solution were serially diluted in 1000 ml distilled water denoted as parts per million (ppm). The treatments are as follows:

Treatment 1 (T1): 4000 ppm, was prepared by diluting 4 ml of acidified chitosan to 1000 ml of distilled water.

Treatment 2 (T2): 6000 ppm, was prepared by diluting 6 ml of acidified chitosan to 1000 ml of distilled water.

Treatment 3 (T3): 8000 ppm, was prepared by diluting 8 ml of acidified chitosan to 1000 ml of distilled water.

Treatment 4 (T4): 10000 ppm, was prepared by diluting 10 ml of acidified chitosan to 1000 ml of distilled water.

Treatment 5 (T5): commercial larvicide, which served as positive control.

Treatment 6 (T6): 1% acetic acid solution, which served as negative control.

Larvicidal Bioassay

The standard procedures for larvicidal bioassay was adapted from the World Health Organization.[40] Complete randomized design (CRD) was used in the study with three replicates. Randomization was done through lottery method wherein each treatment contains batches of 30 third instar larvae of *A. aegypti*. The larvae were transferred by means of a screen loop into a 200 ml disposable container with 100 ml of the treatment. The tested larvae were kept at 25°C with 12 hr dark-light photoperiod and provided with flakes as food. The numbers of dead larvae were counted after 24 hr and 48 hr exposure to the treatments. The larvae recorded are those incapable of rising to the surface or not showing the characteristic of diving reaction when disturbed. Proper disposal of larvae was done after performing the bioassay. All surviving larvae were wrapped in a tissue paper and disposed properly.

Statistical Analysis

Mortality rate was calculated using the equation: % Mortality = [no. of dead larvae / no. of introduced larvae] x 100. Data were pooled from all replicates for analyses. The One-way Analysis of Variance (ANOVA) was used to determine if there is a significant difference in the mortality rate of *A. aegypti* larvae between treatments and *post-hoc* test (LSD) to identify which pairs of means are statistically different after 24 and 48 hr of exposure. The statistical significance (p value) was computed based on 5% level of significance. Regression analysis of log-probit transformed linear curve was determined to calculate the lethal concentration (LC_{50}) using SPSS 17.0 software. The LC_{50} is the specific concentration of the treatment that kills 50% population of the mosquito larvae.

RESULTS

All acidified chitosan treatments have increasing mortality rate of *A. aegypti* larvae as the concentration increases, shown in Figure 1. T4 (83.33±0.06, 96.67±0.06) exhibited the highest while T1 (20.00±0.01, 43.33±0.06) gained the lowest mortality rate of *A. aegypti* larvae after 24 and 48 hr of exposure, respectively. The positive control (T5) showed high mortality rate but not as much as the (T4) highest concentration of acidified chitosan tested. Low mortality rate was observed in negative control (T6).

As presented in Table 1, there is significant difference in mortality of *A. aegypti* larvae among treatments whether exposed for 24 hr or 48 hr. However, the significance can be distinctly observed in the *post-hoc* analysis using

the least significant difference by comparing the mean difference of within-group treatments as shown in Table 2. The mean difference of T4 (-1.67, -0.33) and T3 (1.00, 2.00) of the acidified chitosan is not significantly different from the mortality rate of *A. aegypti* larvae treated with T5 (positive control) after 24 and 48 hr, respectively. In contrast, T1 (4.67, 5.00) and T2 (2.33, 3.33) are significantly lesser than T5. This indicates that among all formulated treatments, the concentration of 10000 ppm acidified chitosan (T4) possessed a high toxicity effect against *A. aegypti* larvae. The mean difference treated with negative control (T6 = 6.67) is significantly lesser compared to the positive control (T5 = 9.00). This shows that acetic acid (negative control) has no significant change in the mortality rate of *A. aegypti* larvae indicating that the larvicidal activity was due to the acidified chitosan. The confidence intervals of T5 showed that mortality rate after 48 hr is significantly higher than compared to 24 hr exposure indicating that the commercial product is also effective against *A. aegypti* larvae. Thus affirming that the concentration of 10000 ppm acidified chitosan (T4) is as good and effective as the commercial larvicide (T5).

Regression line and corresponding mortalities of *A. aegypti* larvae applied with different concentrations of acidified chitosan were established on log-probit linear curve. Figure 2A denotes a lethal concentration (LC_{50}) of 6654.181 ppm that renders 50% mortality after 24 hr. However, a much lesser LC_{50} was estimated at 4942.489 ppm of acidified chitosan that kills 50% population of *A. aegypti* larvae after 48 hr as shown in Figure 2B. With longer exposure, the lethal concentration of acidified chitosan gradually decreased yet larval mortality increases.

DISCUSSION

Results revealed that by increasing the concentration of acidified chitosan, the mortality rate of *A. aegypti* larvae also increases. This indicates to be directly proportional in which high concentration of acidified chitosan leads to high mortality rates. As shown in the results, 10000 ppm of acidified chitosan exhibited the highest toxicity

Table 1: One-way ANOVA of the mortality rate of the treatments against *A. aegypti* larvae.

		Sum of Squares	d_f	Mean Square	F	Sig.
24 hr	Between Groups	141.83	5	28.37	26.87	0.000**
	Within Groups	12.67	12	1.056		
	Total	154.50	17			
48 hr	Between Groups	183.17	5	36.63	59.95	0.000**
	Within Groups	7.33	12	0.610		
	Total	190.50	17			

Note: 5% level of significance; ** Highly significant.

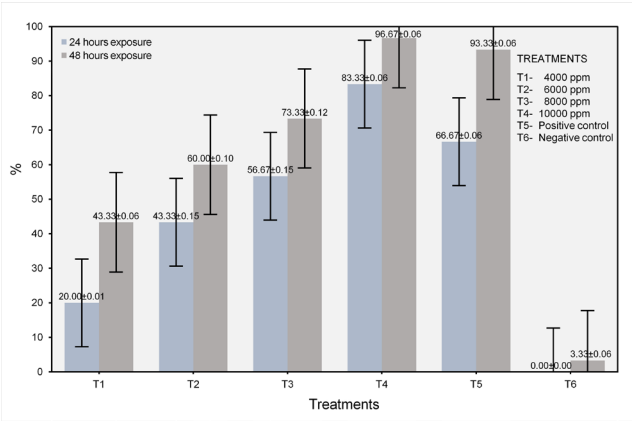

Figure 1: Mortality rate (mean ± standard deviation) of the treatments tested against *A. aegypti* larvae exposed after 24 and after 48 hr.

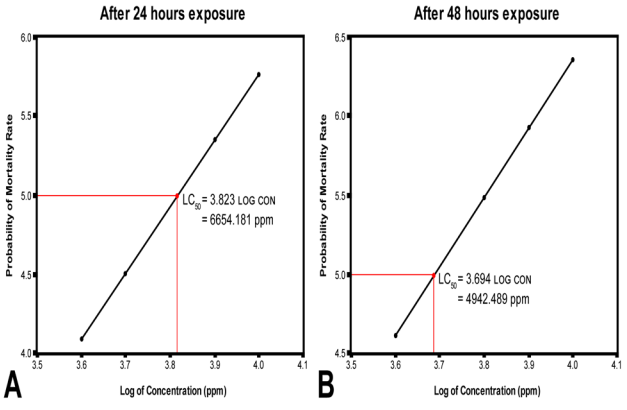

Figure 2: The lethal concentration of the acidified chitosan from crab shell against *A. aegypti* larvae exposed after (A) 24 hr and (B) 48 hr.

Table 2: *Post-hoc* analysis (LSD) of the mortality rate on the mean paired treatments.

Time Exposure	Paired Treatments		Mean Difference	Sig
24 hr	T5	T1	4.67	0.000**
		T2	2.33	0.017*
		T3	1.00	0.256^{ns}
		T4	-1.67	0.070^{ns}
		T6	6.67	0.000**
48 hr	T6	T1	5.00	0.000**
		T2	3.33	0.000**
		T3	2.00	0.009^{ns}
		T4	-0.33	0.611^{ns}
		T5	9.00	0.000**

Note: 5% level of significance; ** Highly significant; * Significant; ^{ns} Not significant. T1- 4000 ppm; T2- 6000 ppm; T3- 8000 ppm; T4- 10000 ppm; T5- commercial larvicide (positive control); T6- acetic acid (negative control).

effect against *A. aegypti* larvae with longer exposure. It appears that the application of chitosan to *A. aegypti* larvae inhibited its feeding activity. This conforms that the immediate effect of insecticides is the reduction of feeding efficiency in the target insects.[41]

It was observed in the present study that the dead larvae of *A. aegypti* exposed to the different concentrations of acidified chitosan showed disintegration of its body tissues. Those larvae that survived after 48 hr of exposure displayed repressed growth and discoloration of the body. Similar findings stated that the effect of chitosan on the epithelium of the larvae midgut of wax moth (*Galleria mellonella*) showed disintegration and have become disorganized when viewed microscopically. Chitosan was detected that it can cause metabolic and synaptic dysfunctions and can alter biochemical physiology.[42]

The third instar larvae of *A. aegypti* treated with acidified chitosan did not undergone molting affecting the metamorphosis of the larvae that inhibits shedding of its cuticle which was followed by larval death. This may suggest that the acidified chitosan has a potential to disrupt metabolic responses and inhibit growth development. Insecticides was reported to have several effects ranging from acute toxicity in larvae and adults through disruption of metamorphosis and interference of reproductive capability of insects, respectively.[43] The biochemical assay of chitosan altered the metabolic pathway in preventing natural excretion of the insect causing a significant increase in urea level.[44] Chitosan can cause significant reduction in total protein contents of the third instar larvae of *G. melleonella* and reduced the activity of alanine aminotransferase which was due to the inhibition of protein synthesis.[45]

The first larvicidal property of chitosan was tested against lepidopterans such as the cotton leafworm *Spodoptera littoralis*, the grey mould *Botrytis cinerea* and the rice leaf blast *Pyricularia grisea*, giving significantly 100% mortality at 0.625 g/kg, with an estimated LC_{50} of 0.32 g/kg.[20] The study of chitosan nanoparticles against third larval instar of *A. aegypti* showed significant results with LC_{50} values of 66.42 mg/L.[46] In the present study, the LC_{50} of acidified chitosan was estimated as 6654.181 ppm and 4942.489 ppm that kills 50% population of *A. aegypti* larvae exposed after 24 and 48 hr, respectively. The high mortality rates of acidified chitosan with longer exposure might be due to some derivatives present in chitosan.

The synthesized derivatives of chitosan are *O*-acyl chitosan, *O*-(butyroyl) chitosan, *O*-(2-methylbutyroyl) chitosan, *O*-(pentanoyl) chitosan, *O*-(heptanoyl) chitosan, *O*-(nonoyl) chitosan and *O*-(decanoyl) chitosan that showed significant reduction in larval length of the third-instar larvae of *S. littoralis*. The *O*-acyl or *N*-(2-chloro-6-fluorobenzyl) chitosan resulted to 100% mortality of larvae with LC_{50} at 0.32 g/kg while the *O*-(decanoyl) chitosan resulted to 64% growth inhibition treated after 5 days.[47] The mechanisms of chitosan might include repellency, disruption of feeding physiology, or chronic toxicity possibly related to larvicidal activity.[48] The larvicidal effect could be due to the denaturation of organelles[49] which decreases membrane permeability and adenosine triphosphate synthesis leading to loss of cellular function and cell death.[50]

CONCLUSION

The acidified chitosan from waste crab shells of *R. ranina* showed toxic effects to *A. aegypti* larvae at higher concentration with longer exposure causing disruptive mechanism on insect's metabolism. This suggest that the utilization of these kind of waste materials can be developed as good alternative larvicides that are environmentally safe and inexpensive. This study warrants to investigate more on the isolation of derivative compounds responsible for the larvicidal property that could be further used in biomedical applications.

ACKNOWLEDGEMENT

The authors are grateful to Mr. Solomon Perez, Entomologist Level II of the Department of Health, for the verification of *A. aegypti* larvae; to the Department of Biological Sciences, College of Science

and Mathematics, Western Mindanao State University for providing the laboratory materials used in the study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DOH: Department of Health; **LC_{50}:** lethal concentration that execute 50% mortality of *A. aegypti* larvae; **OL trap:** Ovicidal/Larvicidal trap; **NaOH:** sodium hydroxide; **ppm:** parts per million; **WHO:** World Health Organization; **ANOVA:** Analysis of Variance; **LSD:** least significant difference.

SUMMARY

The acidified chitosan was extracted from the marine crab shells of *R. ranina* and utilized as larvicide against *A. aegypti*. High mortality rate was observed at increasing concentration of acidified chitosan with longer exposure. Repressed growth and tissue disintegration of *A. aegypti* larvae were noticeable indicating that the acidified chitosan may inhibit growth development and metabolic responses.

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Cite this article: Perez RM, Endaya RJT, Mohammad FS, Sepe MC. Larvicidal Property of the Acidified Chitosan from Marine Crab Shell Wastes against *Aedes aegypti* Larvae. Asian J Biol Life Sci. 2020;9(2):145-51.