RESEARCH ARTICLE

Evaluation of the Anti-inflammatory and Anti-oxidant Activity of Crab Components and Crab Shell in Experimental Rats in Comparison with Dexamethasone and Diclofenac Sodium

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ABSTRACT

Crabs belong to the crustacean family (Decapods crustacean), and their shells contain natural ingredients from which the bioactive compounds are derived. It has been used as folklore medicine in cancer treatment. We investigate the possible anti-inflammatory and anti-oxidant effects for crab shells and whole crabs. Thirty-six rats (150–200 gm) from both sexes were used, divided into six groups, the anti-inflammatory and anti-oxidant activity measured using cotton pellet induce granuloma model. Detection of tumor necrosis factor alpha (TNF α), Interleukin 1 beta (IL-1β), superoxide (SOD), and malondialdehyde (MDA) levels using ELISA Kits. The data analysis by one-way ANOVA followed by the Tukey test. Values are significant at ($p < 0.05$). The results and conclusion showed that the crude material for both crab shell and whole crab have anti-inflammatory and anti-oxidant activity with ELISA analysis.

Keywords: Crab, Crab shell, Cotton pellet induced granuloma, Inflammation, Interleukin 1Beta (IL-1B), Malodialdehyde (MDA), Superoxide dismutase (SOD), Tumor Necrosis Factor alpha (TNF α).

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INTRODUCTION

Inflammation is defined as a protective response of organisms to harmful stimuli that could be invading pathogens or from signals produced endogenously from necrotic cells, results in the elimination of the initial cause of injury, necrotic cells clearance, and start tissue repair.[1] Although it is a beneficial response, if the inflammation is prolonged it could be contributed to the pathogenesis of many diseases such as atherosclerosis, rheumatoid arthritis, and other disease states.[2] The inflammatory response is characterized by five cardinal signs as described by Celsus and Galen which are Rubor (redness), Calor (heat), Tumer (swelling), Dolar (Pain), Functio laesa (Loss of normal functions).[1,3,4] The function of inflammatory response is to eliminate the injurious agents or the by-product produced, limiting their effects and starting damaged tissue repair.[5] If the resolution phase is carried on normally; tissues will be returned to a normal homeostatic state but if the repair phase is not coordinated normally, scarring may occur or may progress to fibrosis or chronic inflammatory state.[6]

The Inflammatory Response

The participants in this pathway are the inducers, sensors, mediators, and affecters. The inducers are the factors that initiate the immune response either exogenous (microbial and non-microbial) or endogenous such as signals from damaged tissue.[7] The sensors are molecules that are activated by inducers called pattern recognition receptors (PRR) that present on the surface of immune cells which able to sense two types of molecules which are pathogen-associated molecular patterns (PAMP) compounds associated with a different pathogen and damage-associated molecular pattern (DAMP) that are associated with the damaged cell of the host.[8] The mediators are molecules that will act to facilitate or inhibit inflammation, increase the pain sensation, activate the affected cells or tissues, and prompt tissue repair. Inflammatory mediators and activated endothelium, facilitate the migration of blood cells mainly neutrophil (extravasation) and plasma with complement factors and antibodies to the inflamed site.[7] The cells involved in the inflammatory response are immune cells such as mast cells, macrophages, neutrophils, dendritic

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cells and non-immune cells such as endothelial cells and fibroblasts.[9] The inflammatory response is accomplished by the production of reactive oxygen and nitrogen species which activate the production of pro-inflammatory cytokines such as TNF α and IL 1 β and chemokine. All these events are to destroy infectious agents and restore tissue homeostasis.[10] Another systems which are involved in this response are the complement, coagulation, and fibrin lytic systems.[11] Finally, this response ceased when the causative agents are removed, repairing the damaged tissue, and restoring body homeostasis.[12]

Types of Inflammation

There are two major types of inflammation acute and chronic inflammation, the differences between them depend on the duration and pathological feature for both of them.[13]

Acute inflammation. Characterized by rapid onset, short duration (hours to days). The stimuli in acute inflammation are varied which could be infectious agents or dangerous foreign bodies and irritants.[9,13] This type of inflammation involves the following steps which are: Vascular events**;** represented by vasodilation of blood vessels leading to increase in blood flow rate with extravasation of plasma proteins and fluids. And Cellular events**;** represented by the migration of immune cells (mainly neutrophils) to the injured site and the production of inflammatory mediators.[13,14] The outcome of acute inflammation is to get rid of the injurious agent, start tissue repair and resolution. If the resolution is failed; then chronic inflammatory diseases may occur such as atherosclerosis and rheumatoid arthritis.[5]

While chronic inflammation lasts for long period from weeks, months, or even years. Examples of stimuli of this type are; failure of elimination of the causative agent in acute type,[15] the persistence of the stimuli, resolution failure, excessive free radical production could be the leading cause of chronic diseases and neurodegenerative disorders.[16] This type of inflammation is characterized by infiltration of macrophages, lymphocytes, vascular proliferation, tissue damage progression, and repair with fibrosis.[14] Marin organism attracts the attention from the researchers for their bioactive compounds isolated from their tissues and organism or for their secondary metabolites.[17]

Crabs

There are a group of animals that belongs to the crustacean family (*decapods crustacean*), their shells contain natural ingredients with different percentages depending on the type and origin of the crab. Their principal components are protein 30 to 40%, calcium carbonate salts 30 to 50%, chitin 20 to 30% and antioxidant compounds such as selenium and carotenoids.[18] The bioactive compounds obtained from marine organisms possess a variety of proteins, peptides, and amino acids which exhibit different pharmacological activities that have been improved such as antifungal, antibacterial, anti-inflammatory, anti-coagulant, and anti-oxidant effects.[19]

Chitin (β-(1-4)-poly-N-acetyl-D-glucosamine) is the most abundant polysaccharide after cellulose, consider as the main component in the exoskeleton of crab and shrimp shells and the walls of fungi and yeast cells.[20] chitin which contains glucosamine in its structure that is used for the treatment of osteoarthritis.[17] Chitin and the compounds derived from it improve its pharmacological activity as anti-bacterial, hypercholesterolemia, anti-inflammatory, anti-oxidant effect and other activities.[21-24] As anti-inflammatory chitin and derived compounds found to reduce NF-kβ expression required for gene expression involved in pro-inflammatory mediator production such as TNFα, IL6, cyclooxygenase, and PGs.[23] Chitosan Oligosaccharides derived from chitin causes inhibition of inflammatory response by reducing the expression of NF-kβ, COX2, iNOS, and pro-inflammatory cytokines.[20] Low molecular weight chitosan produced from chitin is found to enhance the production of anti-inflammatory IL10.[25]

MATERIAL AND METHODS

Drugs and Chemicals

The required standard drugs for this study are Dexamethasone tablet 0.5 mg supplied by ALGORITHM, Lebanon. Diclofenac sodium tablet 50 mg supplied by Acino, Swiss. Olive oil supplied by Zer, Turkey. Diethyl ether supplied by Romil, united kingdom. Formaldehyde 37% supplied by RanReac Applichem, Spain. Povidone Iodine supplied by Poviderm, Turkey. Normal saline was supplied by Pioneer, Iraq.

Samples Collection and Preparations

Originally, crabs were collected from Shat Al-faw, Al Basrah city, Iraq. Crab samples for the present study were collected, washed thoroughly under a stream of water with brushing to remove all the attached sands particles. The internal viscera were removed with further water cleaning. Crabs were dried at 70^{o}C for 6 hours using a Silver Crest German oven. The dried whole crab was broken, smashed, and grinded using a grinder supplied by Bosch German Company for 1.5 minutes at 28000 rpm. The resultant powder was kept in well-sealed polyethylene bags and transferred into the laboratory for the preparation of olive oil suspension.

Another group of crab samples from which the shell was removed, treated by the same procedure for whole crab preparation except for being dried within two hours under the same conditions.

Preparation of Test Compounds and Drug.

Dexamethasone mixture was prepared by grinding of 20 tablets of 0.5 mg dexamethasone with Panasonic, Japan grinder into fine particles were added into a beaker contained olive oil, mixed thoroughly then transferred quantitatively into 200 mL volumetric flask and the volume was completed up to 200 mL with olive oil to have the final concentration of 10 mg/200 mL. The final rat dose of 0.5 mg dexamethasone/kg B.W.[26]

Diclofenac sodium mixture was prepared by taking 4 tablets of diclofenac sodium 50 mg, grinded with Panasonic, Japan grinder into fine particles, added into a beaker contained olive oil, mixed thoroughly then transferred quantitatively into 200 mL volumetric flask and the volume was completed to 200 mL with olive oil to have the final concentration of 200 mg/200 mL. The final rat dose was 10 mg diclofenac sodium/kg of B.W.[27]

Whole crab or crab shell mixtures were prepared by gradual addition of 20 gm of crab or crab shell powder which was previously prepared into a beaker contained olive oil, homogenized for 3 minutes/20000 rpm using Heidolph, German homogenizer. The mixture was transferred quantitatively into a 100 mL volumetric flask and the volume was completed to 100 mL with olive oil. The final concentration of each of the whole crab or crab shells was 20 gm/100 mL olive oil; to administer a dose of 500 mg/kg of rat body weight was given.[28]

Experimental Protocol

Thirty-six Wister rats from both sexes (male and female) weighing (150–200) gm, at developmental stages of (8–10) weeks, were brought from the animal house of the College of Pharmacy, University of Baghdad, Iraq. Rats were maintained on normal conditions of humidity 50 ± 5, temperature (21–25 ± 3)°C, and 12/12 light/dark cycle. They were free to access water but the diet (slandered rat pellets) was withdrawn 12 hours. before the procedure. The experimental protocol was reviewed and approved by the scientific and ethical committee of the College of Pharmacy, University of Baghdad, Iraq.

Cotton Pellet-induced Granuloma

The anti-inflammatory activity evaluated by cotton pellet induces granuloma formation according to Winter and Porter method.[29] The procedure was achieved by cotton pellets weighted 10 ± 1 mg were sterilized by an autoclave at 121°C and 15 Ib pressure. The cotton pellets were implanted on both sides (right and left) subcutaneously into the ventral region (previously shaved and sterilized) in each rat under diethyl ether as anesthesia.

Each of the mixtures of the whole crab, crab shell, diclofenac sodium, and dexamethasone prepared with olive oil, the olive oil free of any addition as control, and cotton pellet induction group were given orally by reusable gavage for 7 days from the day of cotton pellets implantation. On the 8th day, all of the pellets with granuloma were carefully removed and made free from extraneous tissues. The wet pellets were weighted for exudate calculation then dried at 60°C until a constant weight was obtained then the dried pellets were weighed for further granuloma weight calculation. The exudate amount (weight of exudate in mg) was calculated by subtracting the constant dry weight of pellets from the immediate wet weight of pellets followed by the calculation of the percent of exudate inhibition. Then the granuloma tissue formation (dry weight of granuloma in mg) was calculated after deducting the weight of cotton pellet (10 mg) from the constant dry weight of pellet followed by calculation of the percent of granuloma inhibition, then blood sample collection for ELISA analysis. The percent of inhibition is calculated according to the following equations:

$$\text{Exudate inhibition\%} = \left[1 - \frac{\text{Weight of exudate of treated group in mg}}{\text{Weight of exudate of control group in mg}}\right] \times 100$$

$$\text{Granuloma inhibition\%} = \left[1 - \frac{\text{Weight of granuloma of treated group in mg}}{\text{Weight of granuloma of control group in mg}}\right] \times 100$$

Experimental Design

Thirty-six Wister rats were used in this study and divided into six groups:

- *Control group:* Received a single dose of olive oil as vehicle 10 mL/kg.
- *Cotton pellet induction group:* Received a single dose of olive oil 10 mL/kg.
- *Dexamethasone group:* Received a single dose of dexamethasone 0.5 mg/kg with cotton pellets induction.
- Diclofenac sodium group: received a single dose of diclofenac sodium 10 mg/kg with cotton pellets induction.
- *Crab shell group:* Received a single crab shell dose 500 mg/kg with cotton pellets induction.
- *Whole crab group:* Received a single whole crab dose 500 mg/kg with cotton pellets induction.

Enzyme-linked Immune Sorbent Assay ELISA Analysis

ELISA analysis was used for the detection of TNF α, IL 1β, SOD, and MDA levels by using an ELISA kit from Shanghai, China.

Statistical Analysis

All experiment data are expressed as mean ± SEM. Statistical analysis was carried out by using ANOVA followed by the Tukey test. The values are significant at ($p < 0.05$).

THE RESULT

The Anti-inflammatory Effect of Crab Shell and Whole Crab on Exudate and Granuloma Weight and their Percent of Inhibition

The data in Table 1 and Figure 1 shows that both crab shell and whole crab have a minimum inhibitory effect on exudate and granuloma weight with their percent of inhibition as compared with cotton pellet induction group, while dexamethasone shows the maximum inhibitory effect on exudate and granuloma weight and their percent of inhibition followed by diclofenac sodium, whole crab and crab shell, respectively.

Effect of Crab Shell and Whole Crab on Inflammatory Mediators and Antioxidant Activity in Comparison with Dexamethasone and Diclofenac Sodium in Cotton Pellet Induced Granuloma Rat Model

Effect on TNF-α Level in Rat Serum

The data in Figure 2A shows that both crab shell and whole crab groups have a significant reduction in TNF-α level as compared with the cotton pellet induction group. Both groups show a significant reduction better than diclofenac sodium and less than dexamethasone.

Effect on IL-1β Level in Rat Serum

The data in Figure 2B shows that whole crab and crab shell groups have reduced IL1β levels as compared with the cotton pellet induction group. The whole crab shows the maximum reduction effect as compared with dexamethasone and

Table 1: The effect of crab shell and whole crab on exudate weight and granuloma weight with their percent of inhibition

Groups	*mean exudate weight in mg Mean ± SEM*	*Percent of Exudate inhibition %*	*Mean granuloma weight in mg Mean ± SEM*	*Percent of granuloma inhibition %*
Cotton pellet induction group	136.23 ± 0.51	-	23.48 ± 0.40	-
Dexamethasone 0.5 mg group	68.09 ± 0.50 a	50.016	11.42 ± 0.29 a	51.377
Diclofenac sodium 10 mg group	135.15 ± 0.46 b	0.8	21.14 ± 0.17 a, b	9.949
Crab shell 500 mg group	135.77 ± 0.48 b	0.33	23.19 ± 0.16 b, c	1.245
Whole crab 500 mg group	135.28 ± 0.51 b	0.697	23.011± 0.09 b, c	1.99

Each value represents means ± slandered error of means (SEM) (N=6/group). Data were analyzed by ANOVA followed by the Tukey test. Values expressed in non-identical small letters (a, b, c) are significantly different ($p<0.05$).

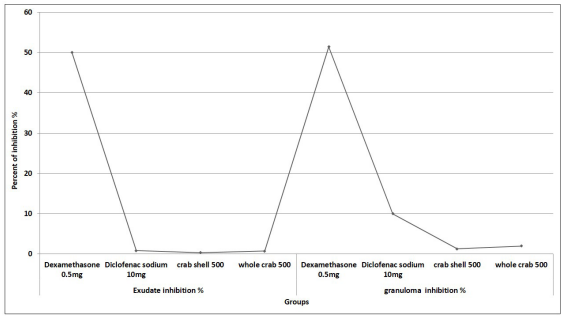

Figure 1: Bar Chart showing the percent of inhibition for exudate and granuloma in different groups in cotton pellet induced granuloma rat model. Each value represents mean ± slandered error of means (SEM) (N=6/group).

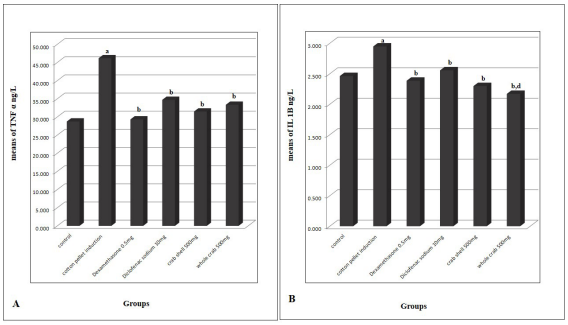

Figure 2(A and B): Bar Chart showing the Tumor necrosis factor TNF-α level in different groups in cotton pellet induced granuloma rat model; (**2B):** Bar Chart showing the Interleukin 1 β level in different groups in cotton pellet induced granuloma rat model. Each value represents the mean ± slandered error of means (SEM) (N=6/group). Data were analyzed by ANOVA followed by the Tukey test. Values expressed in non-identical small. Letters (a, b) are significantly different ($p<0.05$).

diclofenac sodium. While crab shell shows the same level of reduction as compared with dexamethasone, better than diclofenac sodium.

Effect on Super Oxide Dismutase (SOD) Level in Rat Serum

The data in Figure 3A shows that SOD level is reduced in the cotton induction group, while is significantly increased in a crab shell and whole crab groups. The maximum elevated level with dexamethasone followed by diclofenac sodium, crab shell, and whole crab groups respectively.

Effect on Malondialdehyde (MDA) Level in Rat Serum

The data in Figure 3B shows that crab shell and whole crab significantly reduce the level of MDA as compared with the cotton pellet induction group. The level of reduction is mostly the same in all groups.

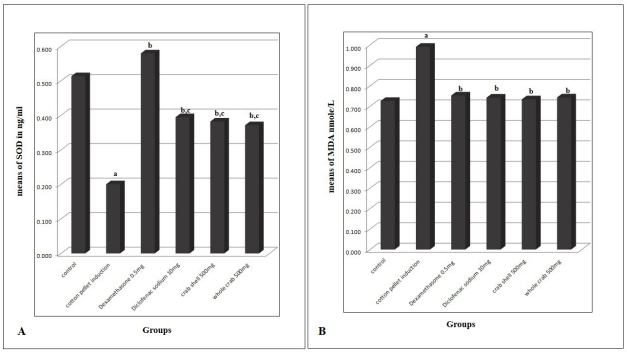

Figure 3 (A and B): Bar Chart showing the (SOD) level in different groups in cotton pellet induced granuloma rat model; (3B) Bar Chart showing the (MDA) level in different groups in cotton pellet induced granuloma rat model. Each value represents the mean ± slandered error of means (SEM) (N=6/group). Data were analyzed by ANOVA followed by Tukey test. Values expressed in non-identical small letters (a, b, c) are significantly different ($p<0.05$)

DISCUSSION

Marin organism attracts the attention from researchers for their bioactive compounds isolated from their tissues and organs or for their secondary metabolites.[17] The biological compounds derived from marine organisms have anti-inflammatory, anti-oxidant, anti-diabetic, anti-coagulant, anti-cancer, and other actions.[19]

The chemical composition of Crabs and crab shells attract our attention in this study due to their chitin content and other bioactive compounds derived from them. Chitin contains glucosamine in its structure which is used for the treatment of osteoarthritis.[17] chitin and a bioactive compound derived from possessing anti-inflammatory, anti-oxidant, anti-bacterial, and other activities.[21,23,24] Selenium and carotenoids that are included in the shell structure have anti-oxidant activity.[30,31]

Anti-inflammatory Effect of Crab Shell and Whole Crab on Cotton Pellet Induced Granuloma

Cotton pellet induces granuloma procedure used for measuring transudate and granuloma formation; the wet weight of pellets related to exudate formation, and extravasation of exudate to

the inflamed site. While granuloma weight correlates with a dry weight of pellets. In chronic inflammatory conditions when the granuloma form there is an accumulation of macrophages, lymphocytes with epitheliod and giant cells derived from macrophages surrounding the foreign body. The inhibitory effect on cotton pellet granuloma is due to the inhibition of fibroblast and neutrophil infiltration and exudation.[32]

Our data in Table 1, Figure 1 about the transudate weight among groups and their % of inhibition shows the maximum reduction with dexamethasone followed by diclofenac, whole crab and crab shell, respectively. The result explains that the crude material of both crab shell and whole crab has the minimum inhibitory effect on exudate weight and percent of exudate inhibition indicates that both groups have minimum effect on leakage of fluid through the capillary endothelial cells as compared with standard drugs.

While for granuloma weight; the data shows the inhibition is higher with dexamethasone followed by diclofenac, whole crab, and crab shell groups. From these data there is a minimum inhibitory effect with the crude material for both crab shell and whole crab groups, this explains both groups have minimum effect on granulocyte infiltration, fibroblast synthesis during granuloma tissue formation. The explanation for these results is that the crude material may require further time to obtain the required effect on exudate and granuloma formation and the percent of inhibition for both.

Effect of Crab Shell and Whole Crab on Inflammatory Mediators

The data from Figure 2A about the effect on TNF-α shows that there is a significant elevation in the level of TNF-α in the cotton induction group and this is in agreement with other studies.[33] The TNF-α level is reduced significantly by both crab shell and whole crab groups, in crab shell, the inhibition is more than the whole crab group. The level of reduction in both groups is less than dexamethasone and more than the diclofenac sodium group.

While **t**he data in Figure 2 B showed that the level of IL-1β was significantly elevated in the cotton induction group and this is in agreement with other studies.[33] The level of IL 1β is significantly reduced with crab shell and whole crab groups and the reduction is more with the whole crab than crab shell group. The level of reduction in the whole crab is better than diclofenac sodium and dexamethasone. While the level of reduction in crab shell is more than diclofenac sodium and mostly the same as the dexamethasone group.

The reduction in the pro-inflammatory mediators (TNF-α, IL 1β, and IL 6) and increased in the level of the anti-inflammatory IL 10 are observed with the compounds derived from chitin but the exact mechanism is not fully understood.[34] Another study result about chitin and chitosan oligosaccharide elucidate the anti-inflammatory effect by inhibiting the phosphorylation of c-Jun N-terminal kinase (JNK) and p38 MAPK pathway.[35] These might be the possible mechanism for the anti-inflammatory effects for the crude crab shell and whole crab groups.

Effect of Crab Shell and Whole Crabs on Antioxidant Activity

Oxidative stress is the imbalance between antioxidant and reactive oxygen species (ROS) species which is well known in chronic inflammatory diseases. SOD is an antioxidant enzyme that acts as a free radical scavenger for O_2, which is responsible for lipid oxidation. The data in figure 3 (A) about the anti-oxidant effect shows that there is a significant reduction in the level of SOD in the cotton induction group and this is in agreement with other results.[36] Superoxide dismutase (SOD) level is significantly elevated in the crab shell and whole crab groups indicates that both groups have anti-oxidant activity. The elevation is most likely to diclofenac sodium and less than dexamethasone level.

Chitooligosaccharides (COS) derived from chitin can up-regulate the antioxidant enzymes including SOD at mRNA level at transcriptional and translational levels through the regulation of Nrf2 activation and phosphorylation of MAPK in ethanol-induced liver oxidative stress this might be one of the possible mechanisms of crab shell and whole crab groups.[37]

While the other possible mechanism on the SOD level could be mediated by selenium which imbedded in crab shell structure.[18] Its deficiency is associated with chronic diseases and neurological disorders. It has detoxification activity against metal poisoning and has a hepatoprotective effect by increasing the level of SOD and GSH enzymes.[38]

Furthermore, the antioxidant effect may be mediated by carotenoids; one of crab shell components.[18] Which has the antioxidant activity that protects cells and tissues from oxidative damage.[39] Carotenoids can react with the free radical molecule to form a more stable one, enhance antioxidant SOD levels and prevent lipid peroxidation.[40]

The MDA is one of the final decompositions of lipid peroxidation and a product of prostaglandin metabolism in the Cyclooxygenase pathway.[41] The data in figure 3 (B) shows that MDA is elevated in the cotton induction group and this is in agreement with other studies,[42] which is significantly reduced by crab shell and whole crab groups, this reduction is mostly similar in all groups.

Oligosaccharides, derived from chitin has a protective effect against oxidative damage induced by H_2O_2 in the serum, liver, and kidney. This results in reducing MDA level.[43] The possible mechanism of oxidative stress may be influenced by COS through down-regulate the phosphorylation of p38, ERK1/2, and JNK in the MAPKs signaling pathway, up-regulation of the Nrf2/ARE signaling pathway to enhance antioxidant response and inhibit ROS production.[44]

Another possible effect on elevated MDA level may be mediated by Selenium.[45] Furthermore, the antioxidant effect may be mediated by carotenoids by scavenging free radicals, increasing antioxidant enzyme levels such as SOD, reducing MDA level, and restoring hepatocyte function in fibrotic liver disease.[46]

CONCLUSION

Our result concerning the anti-inflammatory and anti-oxidant activity with enzyme-linked immunoassay (ELISA) analysis in both crab shell and whole crab groups suggested that the

effect in both groups related to chitin content which might be degraded to produce oligosaccharide and other biologically active products, also related to the synergistic effect produced from selenium and carotenoids that imbedded in crab shell together with active chitin content. There is no major difference between crab shell and whole crab in their effect on anti-inflammatory and anti-oxidant activity in a chronic inflammatory state and the exact mechanism is not fully understood; therefore further investigation on crude material is required.

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