

## Cytotoxic effect of crab shell extracts on different tumor cell lines

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### الخلاصة

السرطان هو حيوان بحري يمتلك خصائص فريدة في سلم التطور ويمتلك العديد من المواد الفعالة ذات الوظائف الخاصة. في دراستنا الحالية تحققنا من الفعل المضاد للسرطان لاربعة مستخلصات مختلفه مشتقه من قشرة السرطان (المائية والكحولية للقشرة المحروقة والقشرة غير المحروقة). اظهرت المستخلصات المائية والكحولية لمسحوق القشرة المحروقه انها تمتلك سمية خلويه عاليه تجاه الخلايا السرطانية وانها تثبط من تكاثرها ولجميع الخطوط المختبرة (خط سرطان الحنجرة وسرطان الخلية العضلية البشرية وسرطانة الغدة اللبنية الفئري) وان هذا التأثير هو تأثير معتمد على مقدار الجرعه والوقت. هذه النتائج تقترح ان لمستخلصات القشرة المحروقه فعلا مضاد للاورام وهي تحتاج الى المزيد من الدراسات.

### Abstract

Crab, a kind of marine animal that has a unique status in evolution, has many bioactive substances with special functions. In the present study, we investigated the antitumor effects of four different extracts derived from crab shell (aqueous and methanolic extracts of burned and normal crab shell powder). Aqueous and methanolic extracts of burned crab shell powder were shown to be cytotoxic and inhibit the *in vitro* proliferation of human laryngeal carcinoma tumor cell lines (Hep-2) and human Rhabdomyosarcoma cell line (RD) and one murine mammary adenocarcinoma tumor cell line (AMN3), in dose and time dependant manner. These results suggest that the burned crab shell powder have an antitumor effect that needs more investigations.

**Key words:** Crab shell, anti-tumor, cytotoxicity

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## Introduction

The study on antitumor activities of marine bioactive substances is an important field in exploiting marine bioactive substances and antitumor drugs (1). Crab, a kind of marine animal that has a unique status in evolution, has many bioactive substances with special functions due to its primitive character. In recent years, many bioactive substances, including clotting factors, protease inhibitors, antibacterial substances, lectins and others, have been found in the hemocytes and hemolymph plasma of horseshoe crab (2, 3, and 4). Tachyplesin, a cationic peptide isolated from acid extracts of the hemocyte debris of horseshoe crab (*Tachyplesus tridentatus*), can rapidly inhibit the growth of both Gram-negative and -positive bacteria at low concentrations (5, 6). Tachyplesin has antitumor effects, Li *et al* (7) investigated the biological effects of tachyplesin on human gastric carcinoma cell line and found that it could alter the malignant morphological and ultrastructural characteristics of it effectively and has certain differentiation-inducing effect on human gastric carcinoma. Chen *et al.* (8) examined a chemically synthesized preparation of tachyplesin that was linked to a RGD sequence and found that this synthetic RGD-tachyplesin could inhibit the proliferation of TSU prostate cancer cells and B16 melanoma cells as well as endothelial cells in a dose-dependent manner *in vitro* and reduce tumor growth *in vivo*. Inhibition of tumor growth was by inducing apoptosis in the tumor cells. Tachyplesin is believed to activate the classic complement pathway to kill tumor cells (9). Crab shells have many natural substances; chitin is one of the most important substances which ranks seconds to cellulose as the most plentiful organic compound on earth. Chemically it is a polysaccharide, which are large molecules strung together like pearls on a strand. Unlike most polysaccharides, chitin has a strong positive charge which allows it to bind to negatively charged surfaces. Chitin and its derivatives; Chitosan, Chitin Oligosaccharides, and Chitosan Oligosaccharides, have many properties that make them attractive for a wide variety of health applications. For example, research indicates that it is anti-bacterial, Anti-fungal and anti-viral, non-toxic and non-

allergic (11, 12). Chitosan synthesized from marine crab shell has anticoagulant activities (12). Kimura and Okuda, (13) found that chitosan might enhance the antitumor activity of cancer chemotherapy drugs and prevent the side effects induced by cancer chemotherapy drugs, such as myelotoxicity, gastrointestinal toxicity and immunocompetent organic toxicity induced by 5-fluorouracil.

In addition, culture-soluble chitosan with low molecular weight might act as an immunomodulator in the intestinal immune system of animals and enhanced the cytotoxic activity of intestinal intraepithelial lymphocyte against tumor (14). Another medical agent found in the crab shell is Glucosamine which used for osteoarthritis sufferers and provide pain relief and to help regenerate damaged tissue in joints (15).

Old manuscript inspired our attention from Islamic folklore medicine talking about using the ashes of crab shell for treatment of cancer patients (16) and from clinical observation about some patients that administered with promising results, this research aimed to explore the antitumor activity of crab shell extracts.

## **Materials and Methods**

### **Crab shell**

All crab shell samples were collected from south of Iraq by Dr.Sharife Al-Alwagy and deposited at the Department of experimental therapy of the Iraqi center for cancer and medical genetic research, Baghdad, Iraq. (Dr.Ahmed M. Al-Shammari)

### **Preparation of extracts**

For each crab sample, shell material removed, dried at room temperature and grounded. Dried powder divided into tow samples: the first extracted directly and the second sample burned in oven at 200 C for 2 hrs leaving burned crab shell material that extracted directly. The dry powder burned and unburned extracted by two ways:

**aqueous:** - about 1 gm of each powder was extracted in 100 ml of water at 100 C by shaking for 5 min. extract were filtered through

Whatman No.1 filter paper and sterilized by filtration through 0.2 Millipore filter (17).

**Methanolic:** Extraction accomplished according (18); the extraction procedure included two steps. In the first, MeOH: H<sub>2</sub>O (9:1) solvent was used, while in the second, a 1:1 ratio of the same solvent was used. In both steps, the period of extraction was 7hrs. The mixture was filtered at the end of each extraction procedure. Filtration facilitated using Whatman no. 1. The two extracts were then combined and placed into open Petri dishes in an incubator at 37°C till dryness

#### **Cell lines and culture medium**

The human Hep-2 (larynx carcinoma), and RD (Rhabdomyosarcoma) and AMN3 (murine mammary adenocarcinoma) were obtained from the Iraqi center for cancer and medical genetic research (ICCMGR-Iraq-Baghdad) and maintained in RPMI 1640 (USbiological, USA) supplemented with 5% calf bovine serum (ICCMGR), 100 units/ml penicillin, and 100 µg/ml streptomycin.

#### **Cytotoxicity assay**

To determine the cytotoxic effect of burned and unburned crab shell extracts treatment, crystal violet assay was conducted as on 96-well plates (Falcon, USA), Hep-2, RD and AMN3 cells were seeded at  $3-4 \times 10^4$  cells/well after 24hr or confluent monolayer is achieved. Cells were treated with each extract at 5000 µg in two fold serial dilution reaching to 39.062 µg/ml. The procedure of adding these therapeutic agents was by adding the extracts for 24, 48 and 72 hrs at 37°C for Hep-2 cell line and for 72hrs for the rest of the cell lines. After the dedicated times, cells were washed with PBS. Cell viability was measured after removing the medium, adding 100 µl of solution of 5mg crystal violet (BDH-England) + 200ml methanol + 50ml formaldehyde 37% and incubating for 20 min at 37°C. After removing the crystal violet solution, cells were washed with water three times. The absorbency was determined on a microplate reader (organon Teknika Reader 230S, Austria) at 492 nm (test wavelength); the assay was performed with modification

in triplicate (19). Endpoint parameters that are calculated for each individual cell line include cell proliferation rate, which is the percentage of control absorbance (22, 23, 20, 21). The inhibiting rate of cell growth (the percentage of cytotoxicity) was calculated as  $(A-B)/Ax100$ , Where A is the mean optical density of untreated wells and B is the optical density of treated wells (24, 25). In addition, the LC<sub>50</sub>, which is the lowest concentration that kills 50% of cells (26) (24).

### **Statistical analysis**

The difference between the response of treated cell and control was analyzed statistically using student's t-test (multiple comparisons).

## **Results**

### **Human larynx carcinoma cell line (Hep-2)**

Showed significant decrease in cell proliferation .Figure 1, 2, 3 and 4 shows the kinetics of proliferation from 24 hrs, 48 hrs and 72 hrs Aqueous and methanolic extract of burned and unburned crab shell treatment. At 5000  $\mu\text{g/ml}$  concentration of aqueous and methanolic extract of unburned crab shell treatment, the growth inhibition (LC<sub>50</sub>) was increased to 66.1 %, 76.4 % and 76 % in 24 hrs, 48 hrs and 72 hrs respectively, While other concentrations was not effective. At 2500  $\mu\text{g/ml}$  concentration of methanolic extract of unburned crab shell treatment, the growth inhibition (LC<sub>50</sub>) was increased to 42 %, 45.3 % and 50.8 % in 24 hrs, 48 hrs and 72 hrs respectively. While burned crab shell treatment was more effective at 625  $\mu\text{g/ml}$  concentration of aqueous extract of burned crab shell, the growth inhibition (LC<sub>50</sub>) was 30.3 %, 0.0 % and increased to 54.4 % in 24 hrs, 48 hrs and 72hrs respectively.

In addition, ethanolic extract of burned crab shell at 625  $\mu\text{g/ml}$  concentration was effective more than ethanolic extract of unburned crab shell, growth inhibition (LC<sub>50</sub>) was increased to 29.3 %, 39.4 % and 57.6 % in 24 hrs, 48 hrs and 72 hrs.

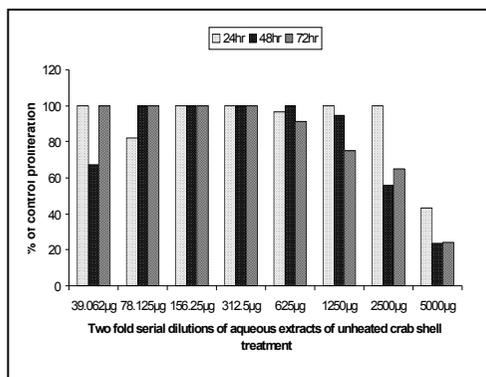


Figure 1. Shows the kinetics of proliferation from 24hrs, 48hrs and 72hrs, aqueous extract of unburned crab shell treatment on Hep-2 cell line.

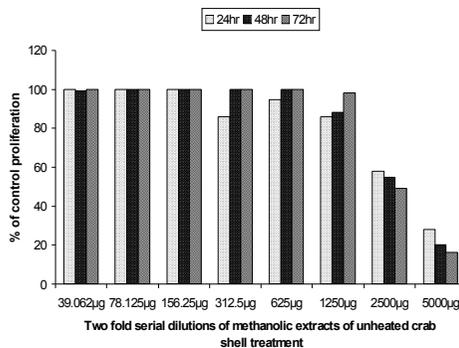


Figure 2. Shows the kinetics of proliferation from 24hrs, 48hrs and 72hrs, methanolic extract of unburned crab shell treatment on Hep-2 cell line.

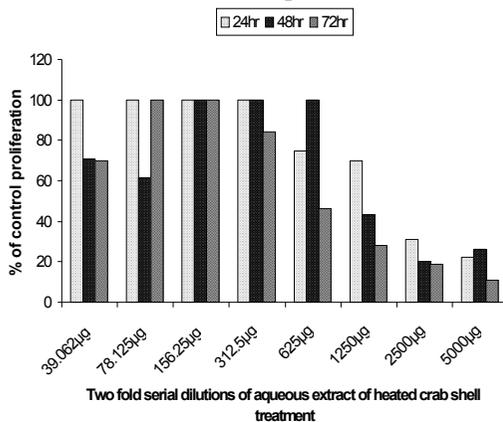


Figure 3. Shows the kinetics of proliferation from 24hrs, 48hrs and 72hrs, aqueous extract of burned crab shell treatment on Hep-2 cell line.

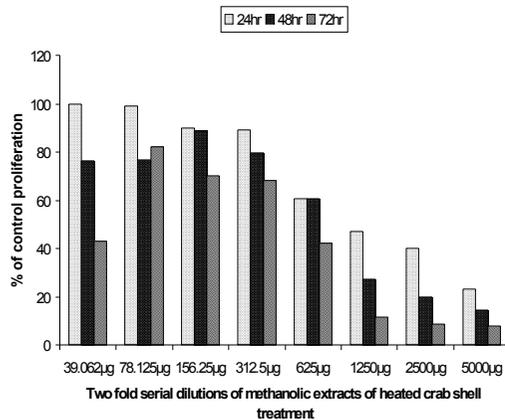


Figure 4. Shows the kinetics of proliferation from 24hrs, 48hrs and 72hrs, methanolic extract of burned crab shell treatment on Hep-2 cell line.

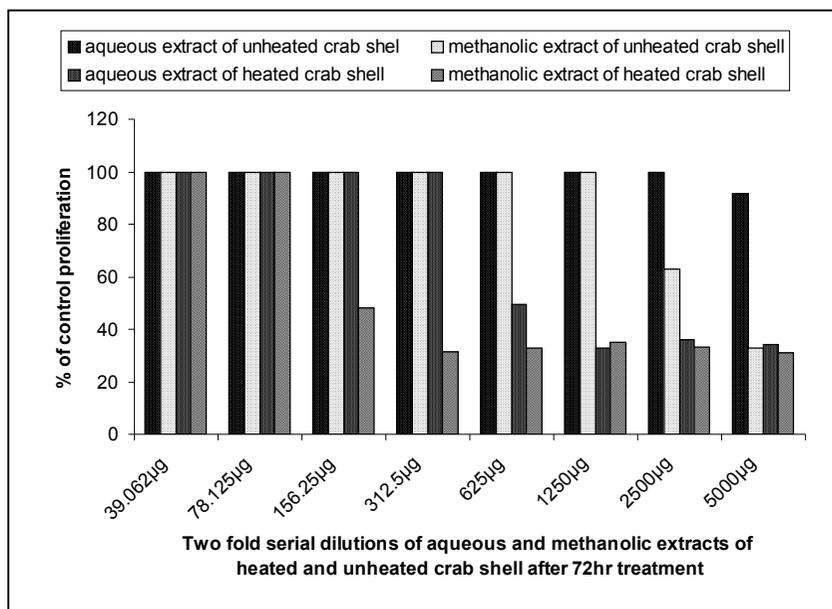
### Human Rhabdomyosarcoma cell line (RD)

Showed a significant decrease in cell proliferation, Figure 5 shows the kinetic of proliferation at 72 hrs water and ethanolic extract of burned and unburned crab shell.

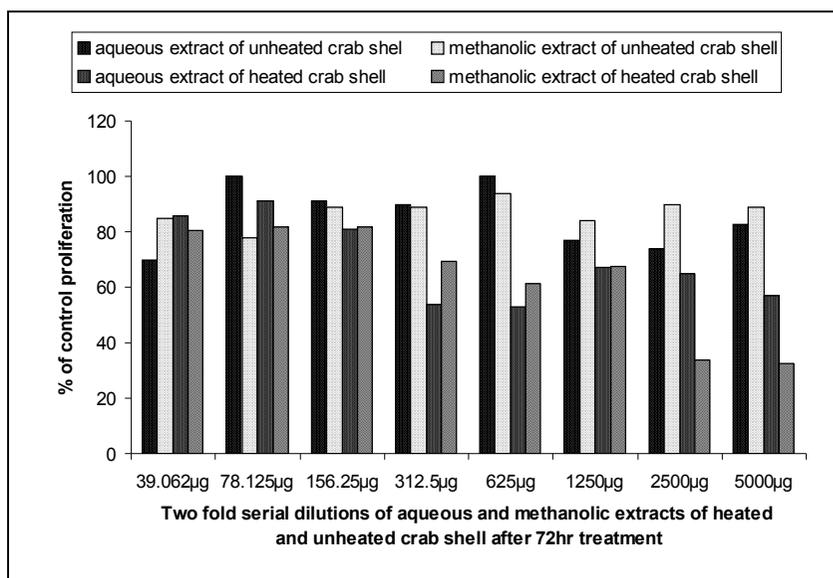
The treatment of RD cells with water extract of unburned crab shell showed no effect at any concentration, while ethanolic extract showed 67.4 % growth inhibition at 5000 µg /ml concentration at 72 hrs. While treatment with water extracts of burned crab shell was more effective and cause 50.6 % growth inhibition (LC 50) at 625 µg /ml in 72 hrs. Ethanolic extract of burned crab shell was also more effective than unburned extracts and water extracts of burned crab shell which was effective at 156.25 µg/ml concentration in 72 hrs by causing 51.8 % growth inhibition (LC 30).

### AMN3, murine mammary adenocarcinoma cell line

Was used to study the effect of extracts on mammary gland tumors, water and ethanolic extracts of unburned crab shell has no effect at all concentrations used, water extract of burned crab shell also has no growth inhibitory effect on AMN3 cells, while ethanolic extracts was effective at 2500 µg/ml (G.I= 66.1 %) (LC 50) in 72 hrs. Figure 6 shows the kinetics of proliferation from 72 hrs extract of burned and unburned crab shell treatment.



**Figure 5.** Shows the kinetics of proliferation from 72hrs, aqueous and methanolic extracts of burned and unburned crab shell treatment on RD tumor cell line.



**Figure 6.** Shows the kinetics of proliferation from 72hrs, aqueous and methanolic extracts of burned and unburned crab shell treatment on AMN3 cell line.

## Discussion

In our study we compare between extracts from burned and unburned crab shell and it appeared from results that extracts from burned crab shell is more effective and have more cytotoxic effect than extracts from unburned crab shell, especially methanolic extract of burned crab shell. To explain the minor antitumor effect of unburned crab shell extracts, it can be explained by presence of chitin and its derivatives, which reported by (13) as an antitumor compound, but this antitumor compound appears less effective when compare with burned crab shell extracts which is more effective. Previous studies reported presence of antitumor compounds in crab, such as Tachyplesin, which is present in leukocytes of the horseshoe crab (5, 6).

Also in crab shell, there is chitin that is mentioned in some research as antitumor compound (14). In previous study by one of our team (16) which mentioned the presence of Ge, Se, Zn, Cr, Mg and Cu in crab shell ashes. Therefore, we suggested or hypothesized formation of new complex during heating in oven for two hrs at 200 C from these minerals and other amino acids or vitamins and polysaccharides that present in crab shell, which need more investigation. Moreover, recent report (27) showed that galactosylated low molecular weight chitosan nanoparticles can bearing positively charged anticancer, doxorubicin (DOX) for hepatocyte targeting and they test it on hepatocellular carcinoma cell line in vitro. In conclusion, we present evidence that extracts from burned crab shell has cytotoxic and growth inhibitory effect on cancer cells and the effect was in dose and time dependent manner and that may suggest potential anti-tumor activity that should be further tested.

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