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***In vitro* anti-tumor effects of hemocyanin isolated  
from *Atergatis roseus* and *Eriphia verrucosa* crabs**

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## *In vitro* anti-tumor effects of hemocyanin isolated from *Atergatis roseus* and *Eriphia verrucosa* crabs

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### ABSTRACT

Hemocyanin (HCY) is a multifunctional glycoprotein, which plays multiple roles in immune defense in invertebrates. HCY from some mollusks can induce potent immune response, while little is currently known about how hemocyanin from arthropods affects tumors. In the present study, *in vitro* cytotoxic effect of HCY from two crab species *Atergatis roseus* and *Eriphia verrucosa* was investigated on two cell lines included hepatocellular carcinoma (HepG-2) and breast cancer (MCF-7). The results showed that HCY exhibited degrees of inhibitory activity against the two cell lines. HCY of *A. roseus* showed the highest cytotoxicity against HepG-2 cell line (IC<sub>50</sub>: 7.7 ± 3.1 mg/ml) while the HCY of *E. verrucosa* showed inhibitory effect (IC<sub>50</sub>: 9.5 ± 2.6 mg/ml) after 72 h. HCY of *A. roseus* and *E. verrucosa* showed their maximum cytotoxicity IC<sub>50</sub> towards MCF-7 at 2.71 ± 1.3 mg/ml and 4.72 ± 1.5 mg/ml, respectively. In Conclusion, we concluded that HCY obtained from *A. roseus* has strong anti-tumor activity against HepG-2 and MCF-7 than HCY obtained from *E. verrucosa*.

**Keywords:** Hemocyanin, multifunctional glycoprotein, *Atergatis roseus*, *Eriphia verrucosa*

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### INTRODUCTION

Invertebrate animals contain the majority of the world's fauna, which are used as experimental animals. The transparent fluid in circulatory system of invertebrates, the hemolymph, has several valuable functions including transports of nutrients, hormones, oxygen and cells (Wang et al., 2015). It contains the respiratory pigment hemocyanin (HCY), which is an oxygen carrier and has a variety of physiological functions specially in mollusca and arthropods such as maintenance of osmotic pressure (Paul et al., 1998), cuticle composition (Paul et al., 1994), regulation of molting (Jaenicke et al., 1999), and regulation of the agglutination of red blood cells and bacteria (Pan et al., 2008). Brouwer (1992) demonstrated that the crustacean HCYS are large copper (Cu) contains protein that consists from 6 subunits with molecular weight of 75 kDa each.

Cancer is one of the most diseases, which cause death of patients. It kills more than 6.7 million people approximately around the world and other 10.9 million new patients were

discovered (Newman et al., 2003). Development of anti-cancer drugs from natural products are ventured through the world nowadays (Vanneman and Dranhoff, 2012). Many researchers have been studied the effect of HCY as antiviral (Zhang et al., 2004; Zanjani et al., 2016), antimicrobial (Jiang et al., 2007) and antifungal (Destoumieux et al., 2001). Moreover, HCY could increase the immune response and protection of mice infected with *Schistosoma japonicum* (Guo et al., 2011). Rizvi et al. (2007) investigated that HCY could stimulate cellular and humoral immune system by interacting of macrophages, CD4 and CD8 T-cells. Interestingly, HCY from two types of marine gastropods acted as potential bio-adjuvant in vaccines (Gesheva et al., 2011).

Keyhole limpet (KL) HCY has a great effect on reduction of recurrence of bladder tumor cells in human (Olsson et al., 1974). Furthermore, KL-HCY has a remarkable role in inhibition of breast, prostate, esophageal, pancreatic, and renal carcinoma (Riggs et al., 2002). HCY from *Helix aspersa* and *H. lucorum* has ant

proliferative and anticancer effects against different malignant cell lines in vitro as breast, leukemia and lymphoma cell lines (Antonova et al., 2014).

*Atergatis roseus* (Ruppell, 1830), the rosy egg crab (Family Xanthidae), has toxic flesh and colonized the eastern Mediterranean by Lesspsian migration through the Suez Canal, Egypt and inhabit coral reefs and rocky substrate from the low tide zone to a depth of 30 meters (Corsini-Foka and Pancucci-Papadobolou, 2010). This alien crab back dated its presence in Egypt through 1989 to 2007 (AbdelSalam and Ramadan, 2016). Another crab species belongs to family Eriphiidae, *Eriphia verrucosa* or warty (yellow) crab (Forsskal, 1775), which lives among stones and seaweeds in the rocky and shallow water to a depth of 15 meters (Rosi and Parisi, 1973). It inhabits the Mediterranean Sea around Alexandria, Egypt (Balss, 1936).

The present study is aimed to address the effect of HCY extracted from two different Egyptian crabs, *A. roseus* and *E. verrucosa* as an anti-tumor agent against two human cell lines including HepG-2 (human hepatocellular liver carcinoma), and MCF7 (breast cancer).

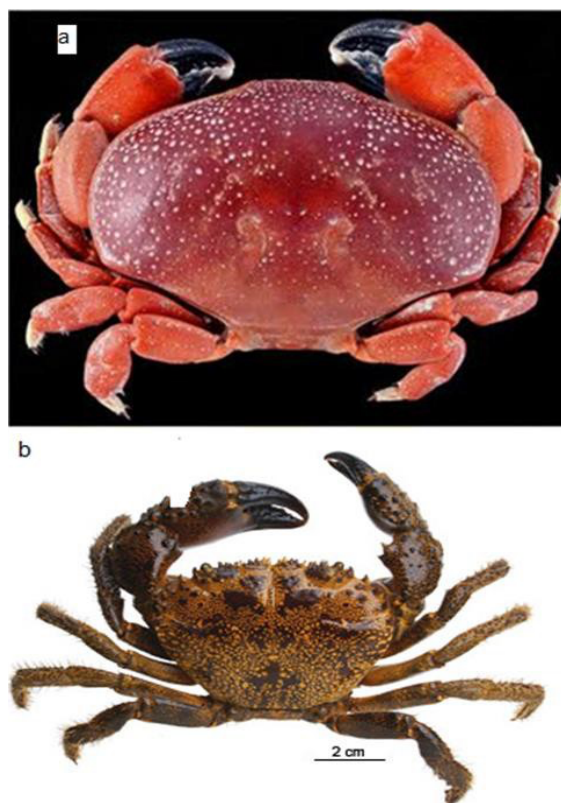
## MATERIALS AND METHODS

### Experimental animals

Specimens were collected early on August 2016 from coastal region in the area from Miami to Abo Qir, Alexandria, Egypt. Two types of crabs were collected from different habitats. *A. roseus* crab (Figure 1A) collected from coral reefs and rocky substrate at 30M depth, while *E. verrucosa* crab (Figure 1B) collected from shallow water 10 M depth among stones. The specimens were transferred in plastic containers to the central laboratory, Zoology department, Faculty of Science, Tanta, Egypt. Crabs were classified and hemolymph were collected for biochemical and screening its anticancer activity.

### Hemolymph collection

The second group of crabs allowed bleeding to collect hemolymph by severing paddle appendage using sharp scissors through meropodite. Hemolymph collected into plastic vials, placed on ice, and allowed to clot.



**Figure 1.** Photomicrograph showing the investigated crab samples *Atergatis roseus* (a) and *Eriphia verrucosa* (b).

The clotted hemolymph was homogenized and centrifuged at 20 000 Xg for 30 min. then, kept the resulting supernatant or serum at  $-4^{\circ}\text{C}$ .

### Determination of total protein and HCY concentrations in hemolymph

The hemolymph serum was diluted with Tris HCL Ca buffer (50 mM Tris/10 mM  $\text{CaCl}_2$ ) PH 8. The dilution was 1:60. Spectrophotometric for total protein and HCY concentration were determined according to Johnson et al. (1984).

### Determination of copper concentration in HCY

To determine Cu concentration, 150 $\mu\text{L}$  of serum was digested using heat block digester after adding nitric acid and hydrogen peroxide. The digested samples were diluted 1:20 with 18.2 dionized  $\text{H}_2\text{O}$  (Li et al., 2012). Concentrations were measured using a NEXION 300 X quadruple (Q) ICP-MS (Perkin Elmer, Ma USA).

### Biochemical composition measurements

Biochemical composition of the muscles was determined. Moisture, ash, total protein, and total lipid were carried out in the Agriculture Research Center (ARC). The crude protein was

determined by the Kjeldahl procedure (AOAC, 1984). Moisture was determined by oven drying at 105°C to constant weight (AOAC, 1990). Total lipid was extracted according to Bligh and Dyer (1959) method. The lipid content was gravimetrically determined. Ash was determined gravimetrically in muffle furnace by heating at 550 °C constant weight (AOAC, 1990).

### Chemical reagents

The reagents RPMI-1640 medium, MTT and DMSO (sigma co., St. Louis, USA), Fetal Bovine serum (GIBCO, UK). Doxorubicin was used as a standard anticancer drug for comparison.

### Cell lines

Two cell lines were used in the experiment; HepG2 (human hepatic cell carcinoma) and MCF-7 (human breast cancer) were obtained from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. Cells were cultured in RPMI-1640 medium with 10% FBS (fetal bovine serum). Antibiotics added were 100 units/ml penicillin and 100µg/ml streptomycin at 37°C in a 5% CO<sub>2</sub> incubator. The cell lines were seeded in a 96-well plate at a density of 1.0 x10<sup>4</sup> cells/well, at 37°C for 48 h under 5% CO<sub>2</sub>. After incubation, the cells were treated with different concentration of HCY extracted from the two crab species and incubated for 24 h, 48 h, and 72 h. After incubation, 20 µl of MTT solution at 5mg/ml was added and incubated for 4 h. 100 µl of DMSO was added to each well and incubate for overnight. Absorbance of the samples was measured using a microplate reader ELISA (EXL 800, USA) at wavelength 570nm. The relative cell viability in percentage was calculated as (A570 of treated samples / A570 of untreated sample) X 100 (Mosmann, 1983).

### *In vitro* cytotoxic assay-MTT assay

MTT Assay is a colorimetric reagent based on the ability of mitochondrial dehydrogenase enzyme present in viable cells to cleave the tetrazolium rings of the MTT dye and form purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells, which are largely impermeable to cell membranes, results in its accumulation in the cells (Subhasree, 2009).

### Determination of inhibition concentration 50 (IC<sub>50</sub>)

According to the FDA, IC<sub>50</sub> represents the concentration of a drug that is required for 50 % inhibition *in-vitro*. In our study, IC<sub>50</sub> is a concentration of HCY at which 50 % of cell population die. IC<sub>50</sub> values were determined from plot of dose response curve between log of compound concentration and percentage cell growth inhibition. Graph was plotted by keeping log concentration of drug on X axis and % Cell growth inhibition or % cytotoxicity Y axis. IC<sub>50</sub> values were estimated as a concentration of HCY at 50 % position on Y-axis. The relationship should be sigmoidal, log concentration of the drug on X-axis and 'response/ measurement' of Y-axis.

## RESULTS

### Total protein, HCY, and copper concentrations in hemolymph.

The data obtained in Table 1 showed significant higher ( $p \leq 0.05$ ) in ratio of total protein and HCY in hemolymph of *E. verrucosa* crab ( $0.15 \pm 0.004$  and  $0.17 \pm 0.03$ , respectively) than their contents in hemolymph of *A. roseus* crab that slower to  $0.044 \pm 0.11$  of total protein and  $0.05 \pm 0.01$  of HCY content. Interestingly, the cu content was correlated positively with HCY concentration, Cu levels were comparable on *E. verrucosa* ( $32.8 \pm 8.5$ ) and *A. roseus* crab ( $29.95 \pm 5.22$ ).

### 3.2. Biochemical composition of crab species muscles

Biochemical composition of the muscles of the two species of crabs was evaluated as shown in Table 2. *E. verrucosa* muscle has higher and significant ( $p \leq 0.05$ ) values of both total protein;  $49.114 \pm 0.21$  g/100g and total lipids;  $23.324 \pm 0.36$  g/100g than *A. roseus* muscles which decreased to  $15.3 \pm 0.13$  and  $2.3 \pm 0.5$  g/100g for total protein and lipids, respectively.

### 3.3. *In vitro* cytotoxicity assay of hemocyanin

The two types of HCY from egg rosy and yellow crabs collected from coastal region of Alexandria, Egypt were tested *in vitro* for their anti-cancer activity on two cell lines HepG-2 and MCF-7 using MTT assay. Viable cells were measured using spectrophotometer at wavelength of 540nm.

**Table 1.** Concentrations of total protein, HCY, and copper in hemolymph of two different marine crabs.

| Parameters            | <i>A. roseus</i> | <i>E. verrucosa</i> |
|-----------------------|------------------|---------------------|
| Total protein (mg/ml) | 0.044 ± 0.11     | 0.15 ± 0.004*       |
| HCY (mg/ml)           | 0.05 ± 0.01      | 0.17 ± 0.03*        |
| % Copper (Cu) mg/l    | 29.95 ± 5.22     | 32.8 ± 8.5          |

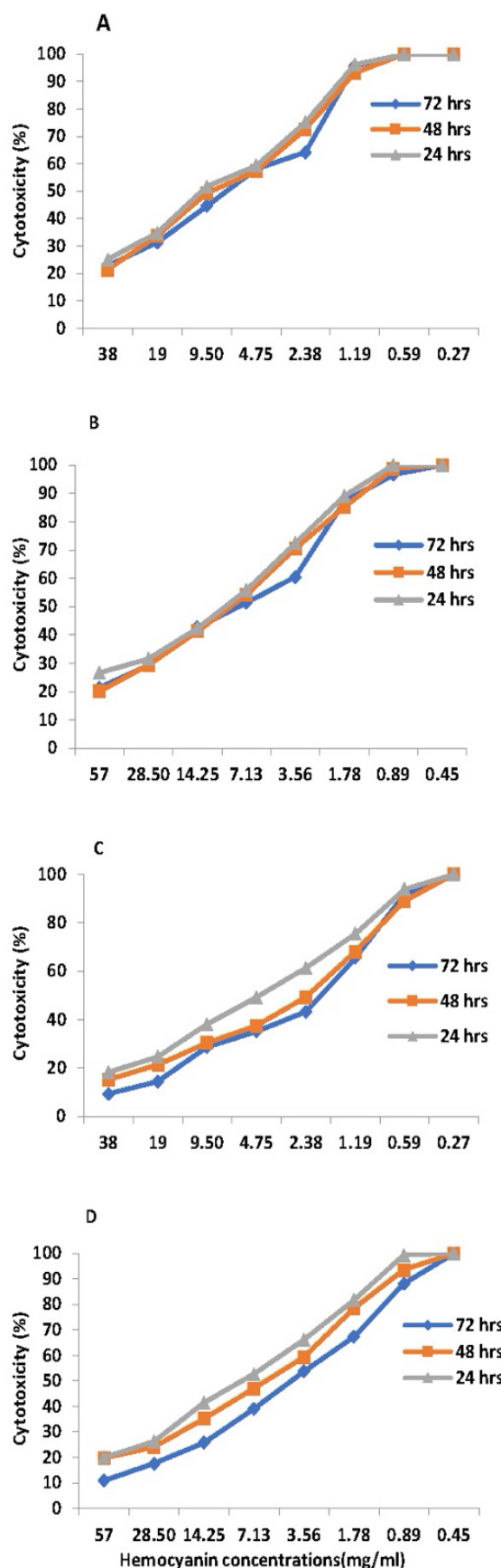
**Table 2.** Biochemical composition of *A. roseus* and *E. verrucosa* crabs.

| Parameters             | <i>A. roseus</i> | <i>E. verrucosa</i> |
|------------------------|------------------|---------------------|
| Total Ash (g/100g)     | 5.7 ± 0.14       | 9.07 ± 0.05         |
| Moisture (g/100g)      | 76.4 ± 0.2       | 68.78 ± 0.403       |
| Total lipids (g/100g)  | 2.3 ± 0.5        | 23.32 ± 0.36*       |
| Total protein (g/100g) | 15.3 ± 0.13      | 49.11 ± 0.21*       |

Measurements were performed and 50 % inhibition of viability or IC<sub>50</sub> were detected of both HCYs type. The data of IC<sub>50</sub> indicated that the maximum inhibitory effect of both HCY types achieved after 72 hr (Figure 2). HCY exhibited degrees of inhibitory activity against the two tested cell lines. As for activity against HepG-2, the highest cytotoxicity obtained by HCY of *A. roseus* showed percentage of IC<sub>50</sub> at 7.7 ± 3.1 mg/ml as compared with HCY of *E. verrucosa* that showed inhibitory effect at IC<sub>50</sub> of 9.5 ± 2.6 mg/ml after 72 h. It is worth mention that MCF-7 showed very high sensitivity against both types of HCY. HCY of *A. roseus* and *E. verrucosa* showed their maximum cytotoxicity IC<sub>50</sub> towards MCF-7 at 2.71 ± 1.3 mg/ml and 4.72 ± 1.5 mg/ml, respectively, after 72 hours. It is obvious from data in Figure 2 that HCY obtained from *A. roseus* has very strong anti-tumor, against the tested human cell lines HepG-2 and MCF-7 than HCY obtained from *E. verrucosa*.

## DISCUSSION

Subphylum crustacean is a largest group of phylum arthropods that includes famous crustacean as crab, lobster, prawn, shrimp, and crayfish. Two types of crabs were collected from Mediterranean Sea in summer; the first one is *E. verrucosa* (Forsk, 1775), it is the only species of family Eriphidia which distributed in eastern and western Mediterranean. Individuals of this species were previously recorded from the Egyptian Mediterranean waters around Alexandria (Balss, 1936), at the

**Figure 2.** Cytotoxicity (%) of *A. roseus* (A) and *E. verrucosa* (B) on HepG-2 and on MCF-7 *A. roseus* (C) and *E. verrucosa* (D).

**Table 3.** *In vitro* cytotoxicity (IC50) of HCY from two types of marine crabs; *A. roseus* and *E. verrucosa*

| HCY (mg/ml)        | MCF-7      |            |           | HepG-2    |             |            |
|--------------------|------------|------------|-----------|-----------|-------------|------------|
|                    | 72h        | 48h        | 24h       | 72h       | 48h         | 24h        |
| <i>A.roseus</i>    | 2.71 ± 1.3 | 3.25 ± 1.6 | 5.1 ± 2.3 | 7.7 ± 3.1 | 8.61 ± 3.7  | 9.71 ± 3.8 |
| <i>E.verrucosa</i> | 4.72 ± 1.5 | 7.27 ± 2.1 | 9.3 ± 2.7 | 9.5 ± 2.6 | 10.25 ± 3.1 | 11.8 ± 3.3 |

\*Significant (p ≤ 0.05)

entrance of Suez Canal (Monod, 1938) and mentioned among Suez Canal's list of true crabs by Holthuis (1956).

It lives among stones and seaweeds in shallow water along rocky coastlines of Alexandria up to depth of 15 meters. The second species collected from Alexandria in the current study, was *A. roseus* which previously collected from Al-Agami and Abo-Qir (Egyptian Mediterranean Sea) by Zenetos et al. (2015) and Abdelsalam and Ramadan (2016). It is one of reef crab of family Xanthidea extending from Red Sea and colonized in eastern Mediterranean through Lesspsian migration (Turkay, 2004). This species inhabits coral reefs and rocky substrate at depth of 30 meters (Yokes et al., 2007). Noguchi et al. (1986), investigated that the flesh of *A. roseus* is toxic due to the toxins of bacteria that symbiosis with that specimen.

The nutritional values of both types of crabs were evaluated in the present study. Obviously, protein content in *E. verrucosa* was 2.2-fold increases than *A. roseus*. lipid content was 9.12-fold increases in *E. verrucosa* than *A. roseus* crab. In deep water crab *Charybdis smithii* the protein contents varied from 59 to 71 % and lipid contents ranged between 6.2 to 8.3 % according to size of the crab. Protein content in claw of blue crab *Callinectes sapidus* that reached to 19.54 g/100g (Kucukgulmez et al., 2006) was less than protein contents of *E.verrucosa* (49.114 g/100g) and more than that in *A. roseus* (15.3 g/100g) in the current study. The same results obtained by krzeczkwesi and Stone (1974) on Snow crab) on Dungeness crab.

Also, Lipid content of *A. roseus* crab was approximately close to its ratio in *Charybdis feriatus* crab that ranged between 0.4 to 1.2 g/100g (Jelin and Panju, 2017). While, lipid contents in both *Ocypode brevicornis* and *Scylla serrata* crabs were smaller than that of the two investigated types of crab in the present study

(Jelin and Kerthika, 2017). Lipid content was relatively low when compared to other decapod crab (Balasubramanian and Suseelan, 2001). The most variable fraction in marine invertebrates was lipid content (Clarke, 1980), also it varied according to the season of specimen collecting (Raymont et al., 1967).

Jelin and Panju (2017) found that the nutritional value of the marine crab was higher than estuary crab *Charybdis feriatus* according to protein and lipid contents. Childress (1971) demonstrated that both Depth and productivity could affect food availability and hence influence biochemical composition, especially lipid and protein ratio, which decreased and as a result water concentration increases with increasing depth. Moreover, Jelin and Kerthika (2017) compared two types of crabs inhibiting two different. Hence, they demonstrated the variable contents of nutritional values because difference in geographical area, environmental factors and available food resources.

Crustacean hemolymph is a circulatory fluid filled its hemocoel. It contains a HCY, a copper based protein (Fredrick and Ravichandran, 2012). HCY has a lot of physiological factors besides oxygen transport in hemolymph of mollusca and arthropods (Wang et al., 2015). It is strikingly that crustacean HCY is a complex structure (Hagner-Holler et al., 2005), it consists from large groups of protein with molecular variances (Decker et al., 2007). Redfield (1934) investigated the HCY concentration in hemolymph of four molluscs as; *Octopus vulgaris*, *Loligo pealei*, *Helix pomatia*, and *Busycon canaliculatum*. Furthermore, the study of Horn and Kerr (1963) has presented the most extensive data on the protein and Cu concentrations in hemolymph of blue crab *Callinectes sapidus*. They found a variation of 10 and 18-fold of both total protein and copper ratio respectively. Hemolymph from abalone *Haliotis corrugata*, *H. cracherodii* and *H.*

*rufescens* had varying values of HCY ranged between 2.03 to 0.0017 g/100ml giving a 900-fold and a 10-fold range between the highest and lowest samples (Pilson, 1962). Over the past twenty years, pharmacological effects of new bioactive compounds in cancer inhibition and treatments have increased. It has shown to have cytotoxic effects against cancer cells without harming normal cells (Katiyar et al., 2009). Two cell lines HepG-2 and MCF-7 were used in this study. Therefore, it is necessary to investigate new bioactive natural compounds which have inhibitory effects against cancer cells without harming normal ones. Senthilraja and Kathiresan (2015) investigated the chemopreventive effects of marine yeast on cancer cells and cause apoptosis with very low toxicity to normal cells *In vitro*.

However, low concentrations of both protein and HCY contents in hemolymph of *A. roseus* crab compared to the second type of crab *E. verrucosa* in the current study, HCY obtained from *A. roseus* crab has very strong anti-tumor, cytotoxic and inhibitory effects against the tested human cell lines HepG-2 and MCF-7 than HCY obtained from *E. verrucosa* crab and MCF-7 cell line was more sensitive to hemocyanin than HepG-2. As shown before, *Vibrio sp* was isolated from xanthid crab *A. floridus* collected from coastal water of Shimoda, Japan (Noguchi et al., 1986). Similarly, non-pathogenic bacteria were isolated from hemolymph of the bivalvian *Galleria mellonella* (Chadwick, 1975). *V. cholera*, *V. vulnificus* and *V. parahaemolyticus* were isolated from the hemolymph and external carapace crab of blue crab *Callinectes sapidus* (Davis and Sizemore, 1982), which may be entered to hemolymph through injuries (Sizemore et al., 1975). Moreover, Noguchi et al. (1986), investigated *vibrio sp* in *A. roseus* crab, this may be the reason for strong cytotoxicity and antitumor effect of HCY of this crab species, however scant protein and HCY values in hemolymph. Wong and Slavec (2015) found that bacteria is well suited to has antitumor effect due to its mobility through the anaerobic environment of tumor environment, cytotoxicity, tumor specificity and therapeutic gene expression (Forbes, 2010), *Salmonella* (Leschner and Weiss 2010), *Clostridium* (Minton 2003), *Bifidobacterium* (Kimura et al., 1980),

*Escherichia* (Yu et al., 2004) and *Listeria* (Quispe-Tintaya et al. 2013) are some of the major genera investigated for their use in cancer treatment. Non-pathogenic and attenuated pathogenic bacteria have a potential role in tumor treatment (Kimura et al., 1980). The anaerobic *Salmonella* is capable of invading both oxygenated and non-oxygenated tumor tissue (Zhao et al., 2005). Invasion of *Salmonella* to solid tumor has been accomplished through auxotrophy, where the recombinant strain is dependent on the tumor microenvironment for essential nutrients not present in sufficient levels in normal tissue (Pawelek et al., 1997). Her and Morlas (2008) demonstrated that *Bacillus* is the most effective bacteria against cancer cells of bladder. So, promising research in biotechnology and molecular technique to engineer bacteria as therapeutic agents.

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