RESEARCH ARTICLE

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_{50} :7.7 ± 3.1 mg/ml) while the HCY of *E.verucossa* showed inhibitory effect (IC_{50} : 9.5 ± 2.6 mg/ml) after 72 h. HCY of *A.roseus* and *E.verucossa* showed their maximum cytotoxity IC_{50} 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.*

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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 30 M 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.

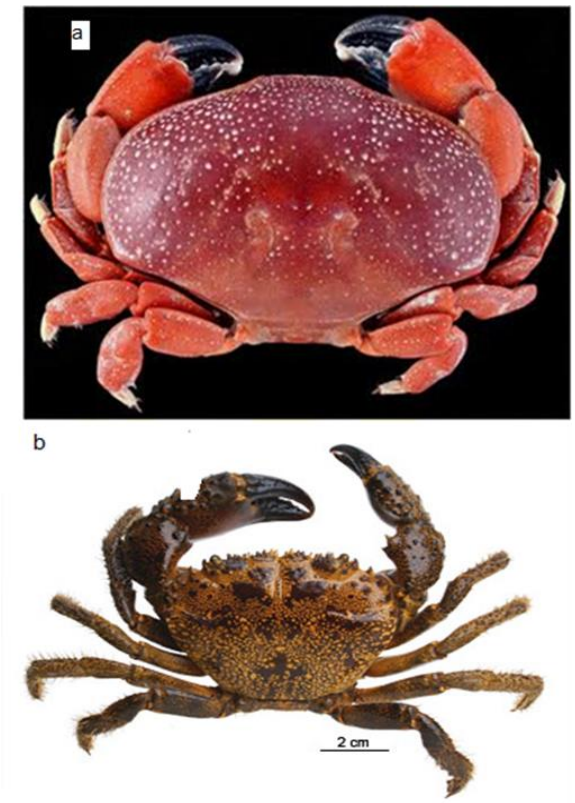

Figure (1): Photomicrograph showing the investigated crab samples *Atergatis roseus (a) and Eriphia verrucosa (b)*

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. The clotted hemolymph was homogenezied and centrifuged at 20 000 Xg for 30 min. then, kept the resulting supernatant or serum at -4°C until use.

Determination of total protein and HCY concentrations in hemolymph

The hemolymph serum was diluted with Tris HCL Ca buffer (50 mM Tris/10 mM CaCL2) 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, 150µLof 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 H2O (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 105ºC 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 ºC 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_2 incubator. The cell lines were seeded in a 96-well plate at a density of 1.0 x10^4 cells/well, at 37◦C for 48 h under 5% CO_2. 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_{50})

According to the FDA, IC_{50} represents the concentration of a drug that is required for 50 % inhibition *in-vitro*. In our study, IC_{50} is a concentration of HCY at which 50 % of cell population die. IC_{50} 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_{50} 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 the X-axis and 'response/ measurement' of the 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. verucossa* crab (0.15 ± 0.004 and 0.17 ± 0.03, respectively) than their contents in hemolymph of *A. roseus* crab that slower to 0.044 ± 0.11 of total protein and 0.05± 0.01 of HCY content. Interestingly, the cu content was correlated positively with HCY concentration, Cu levels were comparable

on *E. verucossa* (32.8 ± 8.5) and *A. roseus* crab (29.95 ± 5.22).

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

Parameters	*A. roseus*	*E. verrucosa*
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

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 ± 0.21 g/100g and total lipids; 23.324 ± 0.36 g/100g than *A. roseus* muscles which decreased to 15.3 ± 0.13 and 2.3 ± 0.5 g/100g for total protein and lipids, respectively.

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

Parameters	*A. roseus*	*E. verrucosa*
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*

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. Measurements were performed and 50 % inhibition of viability or IC_{50} were detected of both HCYs type. The data of IC_{50} 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 IC50 at 7.7± 3.1 mg/ml as compared with HCY of *E.verucossa* that showed inhibitory effect at IC_{50} 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. verucossa* showed their maximum cytotoxity IC_{50} 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 figures 2,3, 4 and 5 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*.

Table (3) *In vitro* cytotoxicity (IC_{50}) of HCY from two types of marine crabs; *A. roseus* and *E. verucosa*

HCY (mg/ml)	MCF-7			HepG-2		
	72h	**48h**	**24h**	**72h**	**48h**	**24h**
A.roseus	2.71 ± 1.3	3.25 ± 1.6	5.1 ± 2.3	7.7 ± 3.1	8.61 ± 3.7	9.71 ± 3.8
E.verrucosa	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 ($p \leq 0.05$)

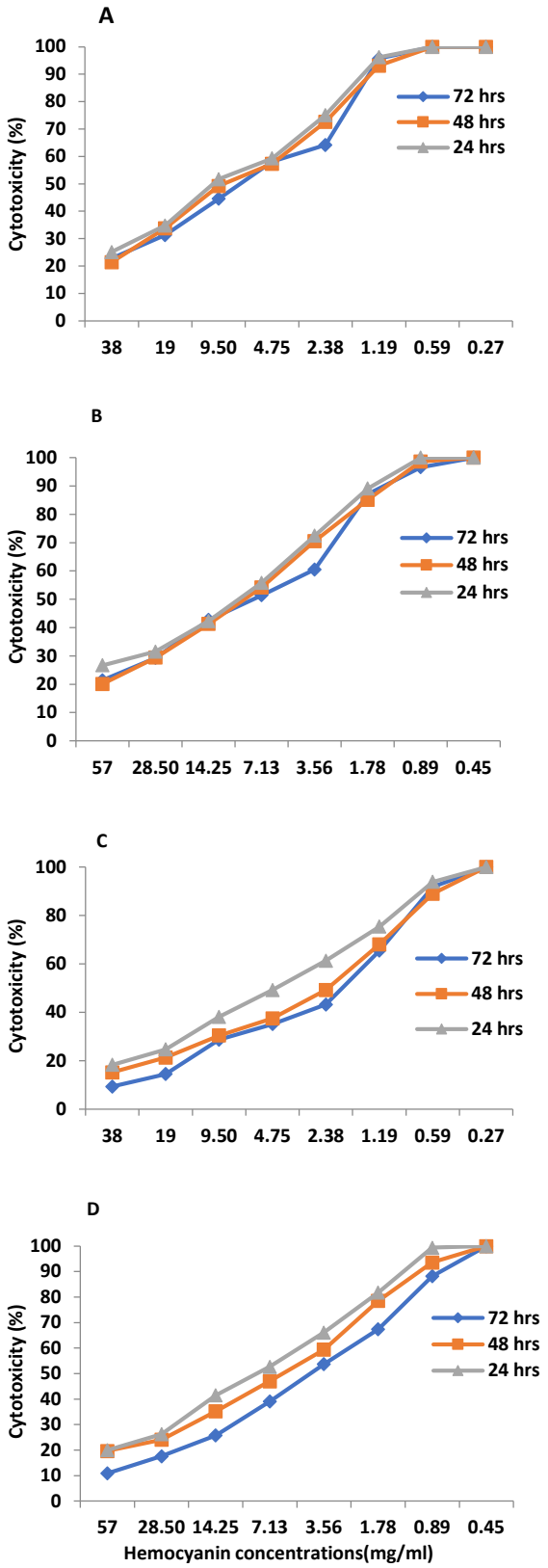

Figure (2): Cytotoxicity (%) of *A.roseus* (A) and *E.verrucosa* (B) on HepG-2 and on MCF-7 *A.roseus* (C) and *E.verrucosa* (D).

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* (Forskal, 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 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 krzeczkowesi 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, pharmalogical 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.verrucossa* 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 *Callincetes 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, cytoxicity, tumor specifity 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.

REFERENCES

Abdelsalam KM and Ramadan SE (2016) Alien crabs in the Egyptian Mediterranean waters. Cah. Biol. Mar; **57:** 363-369.

Andaloro F and Azzurro E (2004) The Sicily channel, a crossroad between Atlantic and Indo-Pacific Worlds. 13th international conference of aquatic invasive species. http://www. icais.org/pdf/21Tuesday/B/tues_b_l_am/Franco_Andaloro. pdf. Cited 25.04.2006

Antonova O, Dolashka P, Toncheva D, Rammensee HG, Floetenmeyer M, Stevanovic S (2014) *In vitro* antiproliferative effect of *Helix aspersa* hemocyanin on multiple malignant cell lines. Z Naturforsch C; **69:** 325–334.

AOAC (1990) Official Methods of Analysis, 15th edn., Association of Official Analytical Chemists, Washington, DC, USA.

AOAC (1984) Official methods of analysis of the Association of Official Analytical Chemistry (14th ed.). Washington, DC: The Association of Official Analytical Chemistry, Inc.

Balasubramanian C and Suseelan C (2001) Biochemical composition of the deep-water crab *Charybdis smithii.* Indian Journal of fisheries; **48(3):** (333–335).

Balss H (1936) Decapoda, The fishery grounds near Alexandria. VII. - Nat. Mem. Fish. Res. Direct. Egypt; **(15):** l-67.

Bligh EG and Dyer WJ (1959). A rapid method for total lipid extraction and purification. Canadian Journal Biochemistry and Physiology; **37:** 911-917.

Brouwer M (1992) Oxygen carrlers as molecular models of allosteric behavior. Adv. comp. environ. Physiol; **13:** 1-16.

Chadwick JS (1975) *In vitro* growth of bacteria in hemolymph of *Galleria mellonella*. Journal of invertebrate pathology; **25(3):** 331-335.

Childress JJ (1971) Respiratory rate and depth of occurrence of midwater animals. Limnology and Oceanography, **16:** 104–106.

Clarke A (1980) The biochemical composition of krill *Euphasia superba* Dana, from South Georgia. Journal of Experimental Marine Biology and Ecology; **43:** 221–236.

Corsini-Foka M, Pancucci-Papadobolou MA (2010) The alien brachyaran crab Atergatis roseus (Decapodes, Xanthidea) in Rhodes Island (Greece). Marine biological association of the united Kingdome; 1-3

Davis J and Sizemore R (1985) Incidence of Vibrio species associated with blue Crabs (*Callinectes sapidus*) Collected from Galveston Bay, Texas.Applied and environmental microbiology; **43(5):** 1092-1097.

Decker H, Hellmann N, Jaenicke E, Lieb B, Meissner U, Markl J (2007) Minireview: recent progress in hemocyanin research. Integr Comp Biol; **47:** 631–644.

Destoumieux-Garzón D, Saulnier D, Garnier J, Jouffrey C, Bulet P, Bachère E (2001) Crustacean immunity: antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge. J Biol Chem; **276:** 47070–47077.

Forbes NS (2010) Engineering the perfect (bacterial) cancer therapy. Nat Rev Cancer; **10:** 785–794.

Fredrick WS and Ravichandran S (2012) Hemolymph proteins in marine crustaceans. Asian Pac J Trop Biomed; **2(6):** 496-502.

Gesheva V, Idakieva K, Kerekov N, Nikolova K, Mihaylova N, Doumanova L and Tchorbanov A. (2011) Marine gastropod hemocyanins as adjuvants of non-conjugated bacterial and viral proteins. Fish Shellfish Immunol; **30:** 135–142.

Guo D, Wang H, Zeng D, Li X, Fan X, Li Y (2011) Vaccine potential of hemocyanin from Oncomelania hupensis against Schistosoma Japonicum. Parasitol Int; **60:** 242–246.

Hagner-Holler S, Kusche K, Hembach A and Burmester T (2005) Biochemical and molecular characterisation of hemocyanin from the amphipod *Gammarus roeseli*: complex pattern of hemocyanin subunit evolution in Crustacea. J. Comp. Physiol; B **175:** 445-452.

Herr HW and Morales A (2008) History of bacillus Calmette-Guerin and bladder cancer: an immunotherapy success story. J Urol; **179:** 53–56.

Holthius LB (1956) Notes on a collection of Crustacea Decapoda from the Great Bitter Lake, Egypt, with a list of the species of Decapoda known from the Suez Canal. Zool. Meded. Deel; **34(22):** 301-330.

Horn EC and Kerr MS (1963) Hemolymph protein and copper concentration of adult blue crab (*Callinectes sapidus rathbun*). Biol. Bull (Woods Hole); **125(3):** 499-507.

Jaenicke E, Föll R and Decker H (1999) Spider hemocyanin binds ecdysone and 20-OH-ecdysone. J Biol Chem; **274:** 34267–34271.

Jelin V and Keerthika (2017) Biochemical composition of marine crab Ocypode brevicornis and estuarine crab *Scylla serrate*. IJNTPS; 2277 – 2782.

Jelin V and Panju A (2017) Comparative biochemical study of the crab *Charybdis feriatus* from marine and estuarine water. IJNTPS; 2277 – 2782

Jiang N, Tan CNS, Ho B, Ding JL (2007) Respiratory protein-generated active oxygen species as an antimicrobial strategy. Nat Immunol; **8:** 1114–1122.

Johnson BA, Bonaventura C and Bonaventura J (1984) Allosteric modulation of *Callinectes sapidus* hemocyanin by binding L-lactate. Biochemistry; **23:** 872-878.

Katiyar RS, Singhvi NR, Kushwaha RV, Ramji Lal, Suryanarayana N (2009) VA-mycorrhizal association in arjuna and jamun trees in forest of Bhandara region, Maharashtra, India. International Journal of Agricultural Sciences; **4(1):** 229-232.

Kimura NT, Taniguchi S, Aoki K and Tsuneo B (1980) Selective localization and growth of *Bifidobacterium bifidum* in mouse tumors following intravenous administration. Cancer Res; **40:** 2061–2068.

King I, Childs MT, Dorsett C, Ostrander JG and Monsen ER (1990) Shellfish: proximate composition, minerals, fatty acid, and sterols. Journal of the American Dietetic Association; **90:** 677–685.

Krzeczkowski RA and Stone FE (1974) Amino acid, fatty acid and proximate composition of snow crab (*Chionoecetes bairdi*). Journal of Food Science; **39:** 386.

Kucukgulmez A, Celik M, Yanar Y, Ersoy B and Cikrikci M (2006) Proximate composition and mineral contents of the blue crab (*Callinectes sapidus*) breast meat, claw meat and hepatopancreas. International Journal of Food Science and Technology; **41:** 1023–1026.

Leschner S and Weiss S (2010) Salmonella-allies in the fight against cancer. J Mol Med; **88:** 763–773.

Li G, Brockman JD, Shih-Wen L, Abnet CC, Schell LA and Robertson D (2012) Measurement of the Trace Elements Cu, Zn, Fe, and Mg and the Ultratrace Elements Cd, Co, Mn, and Pb in limited quantity human plasma and serum samples by inductively coupled plasma-mass spectrometry. American Journal of Analytical Chemistry; **3:** 646-650.

Minton NP (2003). Clostridia in cancer therapy. Nat Rev Microbiol; **1:** 237–242.

Monod T (1938) Decapoda Brachyura. In: Mission Robert Ph Dollfus en Egypte. VIII. Mémoires présentés à l'Institut d'Egypte; **37:** 91-162.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth; **65:** 55-63.

Newman DJ, Cragg, GM and Snader KM (2003) Natural products as sources of new drugs over the period. J. Nat. Prod; **66:** 1022 1037.

Noguchi T, Jeon J K, Arakawa O, Sugita H, Deguchi Y, Shida Y and Hashimoto K (1986) Occurrence of tetrodotoxin and anhydrotetrodotoxin in *Vibrio sp.* isolated from the intestines of a xanthid crab, *Atergatis floridus*. J Biochem; **99:** 311–314.

Olsson CA, Chute R and Rao CN (1974) Immunologic reduction of bladder cancer recurrence rate. J Urol; **111:** 173–176.

Pan JY, Zhang YL, Wang SY and Peng XX (2008) Dodecamer is required for agglutination of *Litopenaeus vannamei* hemocyanin with bacterial cells and red blood cells. Mar. Biotechnol; **10:** 645-652.

Paul R, Bergner B, Pfeffer-Seidl A, Decker H, Efinger R and Storz H (1994) Gas transport in the hemolymph of arachnids I. Oxygen transport and the physiological role of hemocyanin. J. Exp. Biol; **188:** 25-46.

Paul RJ and Pirow R (1998) The physiological significance of respiratory proteins in invertebrates. Zoology; **100:** 298–306.

Pawelek JM, Low KB and Bermudes D (1997) Tumor targeted Salmonella as a novel anticancer vector. Cancer Res; **57:** 4537–4544.

Pilson EQ (1962) Variation of hemocyanin concentration in the blood of four species of Haliotis. The Biological Bulletin; **128(3):** 459-472.

Quispe-Tintaya W, Chandra D, Jahangir A, Harris M, Casadevall A, Dadachova E and Gravekamp C (2013) Nontoxic radioactive Listeria is a highly effective therapy against metastatic pancreatic cancer. Proc Natl Acad Sci; **110(21):** 8668-73.

Raymont JEG, Austin J and Linford E (1967) The biochemical composition o certain oceanic zooplanktonic decapods. Deep Sea Res; **14:** 113 -115.

Redfield AC (1934) The haemocyanins. Biol. Rev; **9:** 175-212.

Riggs DR, Jackson B, Vona-Davis L and McFadden D (2002) *In vitro* anticancer effects of a novel immunostimulant: keyhole limpet hemocyanin. J Surg Res; **108:** 279–284.

Rizvi I, Riggs DR, Jackson BJ and McFadden DW (2007) Keyhole limpet hemocyanin: an effective adjunct against melanoma *in vivo*. Am J Surg; **194:** 628–632.

Rossi and Parisi V (1973) Experimental studies of predation by the crab *Eriphia verrucosa*on both snail and hermit crabs occupants of conspecific gastropod shells. *Bollettino di Zoologia*; **40:** 117–135.

Senthilraja P and Kathiresan K (2015) *In vitro* cytotoxicity MTT assay in Vero, HepG2 and MCF -7 cell lines study of marine yeast. Journal of Applied Pharmaceutical Science; **5(3):** 80-84.

Sizemore RK, Colwell RR, Tubiash HS and Lovelace TE (1975) Bacterial flora of the hemolymph of the blue crab, *Callinectes sapidus*: numerical taxonomy. Applied Microbiology; **29:** 393-399.

Subhasree B, Baskar R, Keerthana RL, Susan R L and Rajasekaran P (2009) Evaluation of anti-oxidant potential in selected green leafy vegetables. Food chemistry; 115: 1213-20.

Vanneman M and Dranoff G (2012). Combining immunotherapy and targeted therapies in cancer treatment. Nat Rev Cancer; **12:** 237–251

Wang J, Zhang FY, Song W, Fang YB, Hu JH, Zhao M, Jiang KJ and Ma LB (2015) Characterization of hemocyanin from the mud crab *Scylla paramamosain* and its expression analysis in different tissues, at various stages, and under *Vibrio parahaemolyticus* infection. Genet. Mol. Res; **14(4):** 16639-16651.

Wang S and Slavec R (2015) Treating cancer with infection: a review on bacterial cancer therapy. Letters in applied microbiology; **61:** 107-112.

Yokes B, Karhan SU and Okus E (2007) Alien Crustacean Decapods from the Aegean Coast of Turkey. Aquatic Invasions; **2(3):** 162–168.

Yu YA, Shabahang S, Timiryasova TM, Zhang Q, Beltz R, Gentschev I, Goebel W and Szalay AA (2004) Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins. Nat Biotechnol; **22:** 313–320.

Zanjani NT, Miranda-Saksena M, Valtchev P, Diefenbach RJ, Hueston L and Diefenbach E (2016) Abalone hemocyanin blocks the entry of HSV-1 into cells: a potential new antiviral strategy. Antimicrob Agents Chemother; **60:** 1013–1012.

Zenetos A, Akel EH, Apostolidis C, Bilecenoglu M, Bitar G, Buchet V, Chalarii N, Corsini-Foka M, Crocetta F, Dogrammatzi A, Drakulić M, Fanelli G, Giglio G, Imsiridou A, Kapiris K, Karachle PK, Kavadas S, Kondylatos G, Lefkaditou E, Lipej L, Mavrič B, Minos G, Moussa R, Prato E, Pancucci Papadopoulou MA, Renda W, Rios N, Rizkalla SI, Russo F, Servonnat M, Siapatis A, Sperone E, Theodorou JA, Tiralongo F and Tzovenis I (2015) New Mediterranean biodiversity records. Mediterranean Marine Science; **16:** 266-284.

Zhang X, Huang C and Qin Q (2004) Antiviral properties of hemocyanin isolated from shrimp Penaeus monodon. Antivir Res; **61:** 93–99.

Zhao M, Yang M, Li X, Jiang P, Baranov E, Li S, Xu M, Penman S and Hoffman RM (2005) Tumor-targeting bacterial therapy with amino acid auxotrophs of GFP-expressing Salmonella typhimurium. Proc Natl Acad Sci USA; **102:** 755–760.