RESEARCH ARTICLE

Assessment of antioxidant, antimicrobial and anticancer activities of carotenoid extracted from *Erugosquilla massavensis* and *Procambarus clarkii* exoskeletons.

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ABSTRACT

The present study was carried out on both marine and fresh water edible crustaceans namely; *Erugosquilla massavensis* and *Procambarus clarkii*, respectively, which are predominant in the local aquatic fauna in Egypt. The present study was conducted to determine the total antioxidant capacity (TAC), total phenolic, total flavonoid and carotenoid content in muscle and exoskeleton of *E. massavensis* and *P. clarkii* crustaceans. The study also evaluate the antioxidant, antimicrobial and antitumor activities of the carotenoid extracted from these species. The results showed that TAC, phenolic and flavonoid contents in both muscle and exoskeleton of *E. massavensis* are higher than those contents in *P. clarkii*. The results showed also that the carotenoid content extracted from the males and females exoskeleton of *E. massavensis* are higher than those of males and females of *P. clarkii*. The antioxidant activity of carotenoid extracted from male and female of *P. clarkii* showed higher activity than those extracted from *E. massavensis*. By contrast, carotenoids extracted from females and males of *E. massavensis* showed higher antimicrobial activity than those of females and males *P. clarkii.* The carotenoid extract shows relatively weak antitumor activity *in vitro* against the Earlich cell lines. In conclusion, both of *E. massavensis* and *P. clarkii* have a bioactive compound with antimicrobial and antioxidant activities.

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INTRODUCTION

The marine life represents almost 80% of the world biota (McCarthy and Pomponi 2004) and used as food, fragrances, pigments, insecticides, medicines etc. More or less 10,000 pharmacologically bioactive compounds have been derived from marine invertebrates such as tunicates, sponges, soft corals, bryozoans, sea slugs and other marine organisms (Fuesetani 2000). Several bioactive compounds are found in soft and hard parts of arthropods such as the flavonoid, chitosan and carotenoids (Fahmy *et al*., 2009).

Erugosquilla massavensis is benthic, marine and predatory crustacean. It has some importance for the fishing industry because it is edible and is used as food sources. It also serves as a pollution indicator since its population changes in relation to changes in the sediment concentration of heavy metals and petroleum (Blasco *et al*., 1999).

Crayfish (*P. clarkii*) is small lobster like freshwater crustacean that has a hard outer skeleton or carapace, which protects the body and makes it rigid. Some bioactive compounds in the hard part of *P. clarkii* as astaxanthin which introduced in the food of laying hens as a source of carotenoid astaxanthin (Pérez-Gálvez *et al*., 2008).

Polyphenols are among the most widespread class of secondary metabolites in nature. Most polyphenols arise from a common origin; the amino acids phenylalanine or tyrosine. These amino acids are delaminated to cinnamic acids, which enter the phenylpropanoid pathway. These molecules present antioxidant, antimutagenic, antiviral, antibacterial, algicidal, antifungal, insecticidal and estrogenic activities that may serve to protect the organism from competing ones in their biological environment (Yang *et al*., 2001; Lacikova *et al*., 2009).

Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action. Some evidence suggested that the biological actions of these compounds are related to their antioxidant activity (Koleva *et al*., 2002; Pourmorad *et al*., 2006). Antioxidants are important species which possess the ability to protect organisms from damage caused by free radical-induced oxidative stress (Choi *et al*., 2002). The antioxidant activity of phenolic is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators **(**Ozsoy *et al*., 2009).

Carotenoids are a class of fat-soluble pigments found principally in plants, algae, photosynthetic bacteria and animals. They are responsible for the colours in fruits, vegetables, fish, and crustaceans. Although animals are incapable of producing carotenoids, they incorporate carotenoids from their diets and can be a source of vitamin A (Britton *et al*., 1995). Carotenoids have been studied for chemopreventive properties and they constitute the main group of pigments found in aquatic animals (Meyers, 2004) producing colours from yellow to dark red (Latscha, 1989). The main chain of their chemical structure is 40 carbons long, highly unsaturated, inflexible, and easily oxidizable (Olson, 1994). To date, more than 750 carotenoids have been identified in nature and over 250 of those are from the marine origin (Maoka, 2011).

Another major feature of carotenoids is the protection of low density lipoproteins oxidation LDL against oxidation (Bub *et al*., 2000). In addition, carotenoids have been shown to inhibit in vivo lipid peroxidation processes (Seppanen and Csallany, 2002). The antioxidant activity of some carotenoids, during radical peroxide-induced cholesterol oxidation, was investigated by Palozza *et al*. (2008), showing that carotenoids exerted a significant antioxidant activity. It is interesting to note that, carotenoids are being considered as important protective molecules in gastric disorders. A high intake of carotenoids prevents the development of disorders caused by *Helicobacter pylori* (Molnár *et al.,* 2010). Carotenoids in the carapace of crustaceans exist as both free and esterified forms. The principal carotenoid in crustaceans is astaxanthin (Matsuno, 2001). In crustaceans, astaxanthin exists as carotenoproteins such as crustacyanin, and exhibits purple, blue, and yellow colours. The present study aimed to determine the concentration of total antioxidant capacities, phenolic, flavonoid and carotenoid contents in both of male and female *E. massavensis* and *P. clarkii* muscles and exoskeleton. The study also aimed to determine the antioxidant, antimicrobial and anticancer activities of the carotenoid extracted from the exoskeleton of *E. massavensis* and *P. clarkii*.

Materials AND METHODS

Study areas and samples collection

Mantis shrimp E. massavensis samples were obtained from the Mediterranean Sea at Alexandria from Eastern Harbour (Alex, Egypt). Crayfish *P. clarkii* samples were collected from the River Nile, Mahmodya Canal (Egypt). The samples were collected at night using commercial trawlers.

E. massavensis (Linnaeus, 1758) species was identified based on the morphological

description provided by Makarov (1971). The *P. clarkii* (Girard, 1852) species was identified based on the morphological description provided by Saad and Emam (1998).

Determination of total antioxidant capacity

Total antioxidant capacity (TAC) of the wet homogenate of muscles, and carapaces of selected samples were assayed by the colormetric method using the available commercial kit (Bio - diagnostic, Egypt) according to the method described by Koracevic *et al.* (2001**)**.

Determination of total phenolic and flavonoid contents

The samples were first ground to fine powder. For water extraction, 0.5 g of the fine powder was extracted with 10 ml of ultra-filtered water at 100 °C for 30 min in a water bath. For methanol extraction, 0.5 g of the powder was extracted with 10 ml of 80% methanol at 40 °C for 24 h. The samples were then cooled down to room temperature and centrifuged at 4500 rpm for 15 min (Wolfe *et al*., 2003; Ordonez *et al*., 2006).

The total phenolic content of soft and hard part extracts was determined using the Folin-Ciocalteu reagent. The reaction between Folin-Ciocalteu and phenolic compounds results in the formation of a blue colour complex (Wolfe *et al*., 2003). Total flavonoid content was determined using aluminum chloride ($AlCl_3$) according to a known method based on the formation of a complex between $AlCl_3$ and the carbonyl and hydroxyl groups of flavones and flavonols that produce a yellow colour, using quercetin as a standard (Ordonez *et al*., 2006).

Extraction of carotenoids from exoskeletons

Carotenoids were extracted from shells of both male and female of investigated samples according to Chen and Yang (1992). Exactly 15 g of shrimp shell powder was mixed with 150 ml of acetone in a flask and shaken for 30 min. The filtrate was collected using a suction pipe, and the residue was extracted with 150 ml of acetone again. Likewise, the residue was extracted with 150 ml of petroleum ether twice until it became colorless. All of the extracts were combined in a flask, and 600 ml of 10% sodium sulfate solution was added and shaken for 1 min. After which the mixture was left in the dark until two layers were formed. The supernatant was collected, and the residue was repeatedly extracted with 150 ml of petroleum ether four times. All of the extracts were combined, evaporated to dryness, carotenoid as astaxanthin exists naturally in deep orange to red colour. To determine the amount of total carotenoids extracted, UV-visible spectrophotometry was used for a spectral window between 380 and 750 nm, in triplicate. Carotenoid concentration was obtained using the Lambert-Beer law and for calculation purposes the following equation was applied to the absorbance values:

$$\text{Total carotenoid } \mu g/g = \frac{(\text{Absorbance/e x-molecular mass} \times 1000 \times \text{sample volume (ml)})}{\text{Sample dry weight (g)}}$$

The specific optical extinction coefficient e^{1}_{1cm} of $124000_{(astaxanthin)}$ at 460 nm was used (Buchwaldt and Jencks, 1968) in conjunction with a molecular mass of 596.84 (astaxanthin). Absorbance measurements were performed in triplicate and the values previously averaged were applied in the above equation (Minguez and Mendez, 1993).

Determination of DPPH scavenging activity

The antioxidant activity of the extracts of male and female *E.* massavensis and *P. clarkii* and the standard ascorbic acid were assessed on the basis of the radical scavenging effect of the stable DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical. The radical scavenging effects of antioxidants on DPPH are due to their hydrogen donating ability which causes an absorbance drop at 517 nm. Serial dilutions (5, 12.5, 25 and 50 µg/ml) of each compound were measured by the same assay to obtain IC_{50} (the amount of compound that gives the half-maximal response). In the DPPH radical scavenging, antioxidants react with the DPPH radical, which is a stable free radical and exists naturally in deep violet colour, to turn into a yellow coloured (diphenyl picryl hydrazine). The degree of discolouration indicates the radical scavenging potential of the antioxidant (Williams *et al.,* 1995).

MIC determination of antimicrobial activity

Half-fold serial dilutions were made for selected complexes in order to prepare concentrations of 6.25, 12.5, 25, 50 and 100 mg/ml in distilled water, zero concentration was considered as a negative control. A previously prepared pure spore suspension of each test microorganism (0.5 ml of about 10^6 cells/ml) was mixed with 9.5 ml of each concentration in sterile test tubes, incubated at 27 °C for 3 days for fungi (*Candida albicans*) and at 32 °C for 24 hours for bacteria (*Escherichia coli* and *Staphylococcus aureus*) then optical density of growth was measured by spectrophotometer (Optima SP-300, Japan) at 620 nm for each incubated mixture, results were represented graphically, and MIC was recorded for each tested material (Shadomy *et al*., 1985).

Determination of the anticancer activity of carotenoid extracted from exoskeleton.

The cytotoxicity of the extracts was tested against Ehrlich Ascites Carcinoma cells (EAC) cell lines by SRB assay, according to Vichai and Kirtikara (2006). Briefly, the adherent cells were collected after trypsinization using 0.25% Trypsin-EDTA then washed twice and plated in 96-well plates at 1000 to 2000 cells/well. Cells were exposed to different extracts for 72 h and subsequently fixed with 10% trichloroacetic acid (TCA) for 1 h at 4°C. After several washing using distilled water, cells were exposed to 0.4% SRB solution (dissolved in 1% glacial acetic acid) for 10 min in dark place. A 1% glacial acetic acid was used to wash the plates several times. After drying overnight, Tris-HCl was used to dissolve the SRB-stained cells and colour intensity was measured at 570 nm with a microplate reader. The results were linear over a 20-fold range of cell numbers and the sensitivity is comparable to those of fluorometric methods.

Statistical analysis:

Statistical analysis data were expressed as Mean ± SD. Analysis of paired t-test was used to determine the level of significance at $p \leq 0.05$. All statistical analysis was performed using the SPSS (software version 17.0).

RESULTS

Antioxidant capacities in the muscles and exoskeleton of male and female of *E. massavensis* and *P. clarkii*

As shown in Figure 1 the highest antioxidant contents are recorded in the muscles of female and male *E. massavensis* ($p < 0.05$) compared with other investigated hard and soft tissues. While hard part of *P. clarkii* shows a minimal content

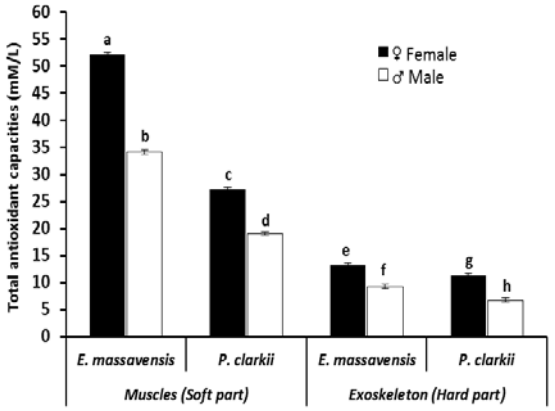

Fig. (1): Total antioxidant capacities in muscles (soft part) and exoskeleton (hard part on dry weight) of male and female *E. massavensis* and *P. clarkii.*

Total phenolic compounds

The highest content of phenolic compound was recorded in the muscles of *E. massavensis* methanol extract ($p < 0.05$) followed by the females exoskeleton methanol extract of the same species ($p < 0.05$). While the minimal content was recorded in the females exoskeleton water and methanol extract of *P. clarkii*

(Fig.2)

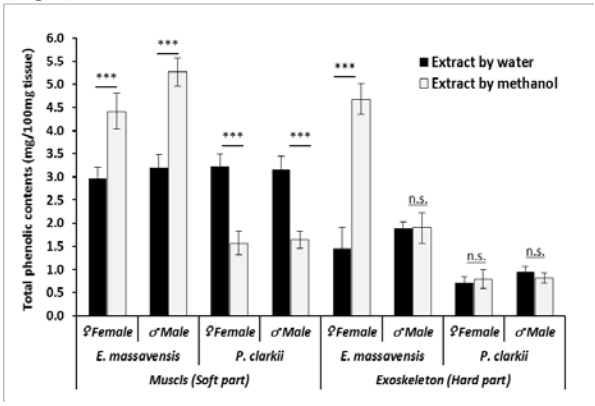

Fig. (2) Total phenolic contents in muscles (soft part) and exoskeleton (hard part on dry weight)

extracted by water or by methanol in male and female *E. massavensis* and *P. clarkii*

Total flavonoid contents

The highest contents of flavonoids were recorded in methanol extract of *E. massavensis* males and females exoskeleton ($p < 0.05$) and ($p < 0.05$), respectively (Figure 3). While the lowest content is recorded in methanol extract of of male P. clarkii muscles ($p < 0.05$).

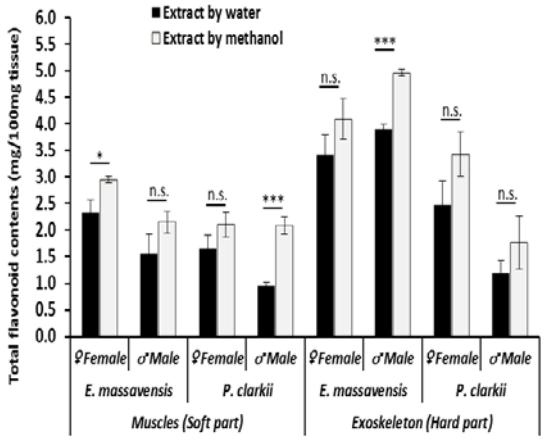

Fig. (3) Total flavonoid contents in muscles (soft part) and exoskeleton (hard part on dry weight) extracted by water and by methanol in male and female *E. massavensis* and *P. clarkia*

Total carotenoid contents in exoskeletons

The concentration of carotenoid from the exoskeleton of both male and female of *E. massavensis* and *P. clarkii* are shown in figure 4. The concentration of carotenoids (as astaxanthin) extracted from female *E. massavensis* (4.46 ± 0.02 µg/g) was more than male *E. massavensis* (3.66 ± 0.02 µg/g) by 1.22 fold. While, the concentration of carotenoids of male *P. clarkii* (2.99 ± 0.02 µg/g) is more than female *P. clarkii* (2.59 ± 0.01 µg/g) by 0.87 fold.

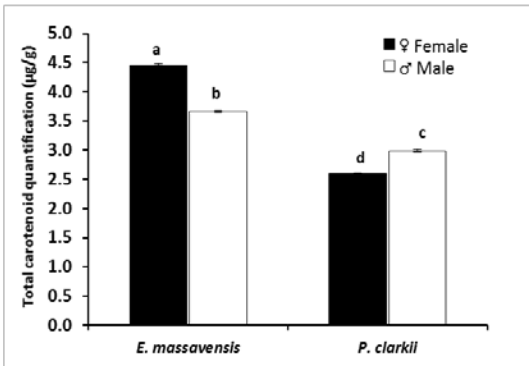

Fig. (4): Total carotenoid contents in exoskeleton (hard part on dry weight) of male and female *E. massavensis* and *P. clarkii*

Antioxidant effect of the carotenoid extract

The results of the antioxidant effect are showed in Table 1. and Figure 5. Carotenoid that extracted from the exoskeleton of male and female *P. clarkii* showed good results as an antioxedent activity using DPPH scavenging method, However, males and females *E. massavensis* showed a relatively weak antioxidant activity.

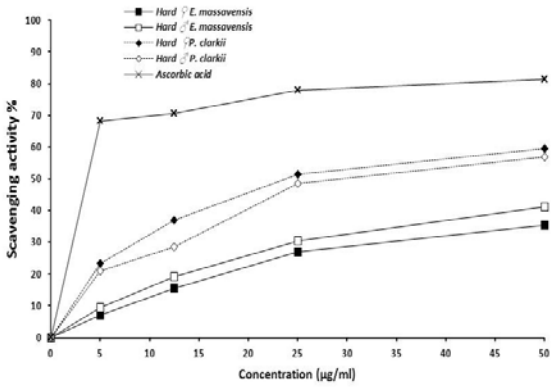

Fig. (5) Antioxidant effect of carotenoid showing scavenging activity %

Table (1): Antioxidant effect of carotenoid at different concentration showing scavenging activity (%).

Extract	Sex& species	Concentration (µg/ml)			
		5	12.5	25	50
Carotenoid as astaxanthin from exoskeleton (hard part)	Hard♀ *E. massavensis*	7.18	15.56	26.94	35.43
	Hard♂ *E. massavensis*	9.58	19.16	30.53	41.31
	Hard ♀ *P. clarkii*	23.35	37.12	51.49	59.68
	Hard ♂ P. clarkii	20.95	28.74	48.50	56.98
Reference compound (Ascorbic acid)		68.26	70.65	77.84	81.43

Antimicrobial and antitumor activities

The results of the antimicrobial activities are shown in Tables 2 and 3. Carotenoid from the exoskeleton of female and male *E. massavensis* showed a relatively high antibacterial activity against both *Escherichia coli* and *Staphylococcus aureus*, as well as high antifungal activity against *Candida albicans*. Carotenoid from the exoskeleton of male *P.*

clarkii showed antibacterial and relatively medium antifungal activity. The antitumor activities of extracted compounds against Ehrlich carcinoma are shown Table (4). Carotenoid as astaxanthin from the exoskeleton of both male and female *E. massavensis* and *P. clarkii* showed relatively weak antitumor activities.

Table (2): antimicrobial activity of carotenoid extract of female and male *E. massavensis* and *P. clarkii* on *C. albicans*, *E. coli*, and *S. aureus*.

Extract	Sex & species	*C. albicans*	*E. coli*	*S. aureus*
Carotenoid as astaxanthin from hard part	Hard ♀ *E. massavensis*	1.6	1.5	1.6
	Hard ♂ *E. massavensis*	1.8	1.9	1.8
	Hard ♀ *P. clarkii*	0.9	0.9	0.9
	Hard ♂ *P. clarkii*	1.5	1.2	0.9

Table (3): Antimicrobial effects of carotenoid extract of male and female *E. massavensis* showing against different sensitive microorganisms *C. albicans*, *E. coli*, and *S. aureus.*

Microorganism	Percentage of surviving cells (% Optical density)						Extract
	Concentration (mg/ml)						
	0.0	6.25	12.5	25	50	100	
Candida albicans	100	65	67	31	30	30	Hard ♀ *E. massavensis*
	100	69	29	28	27	26	Hard ♂ *E. massavensis*
Escherichia coli	100	73	61	34	36	34	Hard ♀ *E. massavensis*
	100	82	28	28	27	28	Hard ♂ *E. massavensis*
Staphylococcus aureus	100	58	49	29	28	27	Hard ♀ *E. massavensis*
	100	73	31	30	31	32	Hard ♂ E. massavensis

Table (4): Antitumor effect of carotenoid extracts of female and male *E. massavensis* and *P. clarkii* on Earlich carcinoma.

Extract	Sex & species	Live	Dead	Total	% viability
Carotenoid as astaxanthin from exoskeleton (hard part)	Hard female *E. massavensis*	1.3×10^6	8.5×10^5 (39%)	2.2×10^6	61%
	Hard male *E. massavensis*	1.6×10^6	6×10^5 (27%)	2.2×10^6	73%
	Hard female *P. clarkii*	2×10^6	1.6×10^6 (44%)	3.6×10^6	56%
	Hard male *P. clarkii*	1.7×10^6	4×10^5 (19%)	2.1×10^6	81%
Control			3%		97%
Reference drug Doxorubicin (Dox)			89%		11%

DISCUSSION

Marine and fresh water edible crustaceans, *E. massavensis* and *P. clarkii*, are important species in aquatic fauna in Egypt. The secondary metabolites from marine and fresh water crustaceans have gained importance in the pharmaceutical and pesticide industries. Crustaceans are one of the important natural sources of secondary metabolites such as carotenoids, phenolic compounds and flavonoid. The antioxidant capacity of these compounds has been related to the prevention of several diseases including cancer, coronary heart diseases, inflammatory disorders, neurological degeneration, and ageing (Wollgast and Anklam 2000; Zhou et al., 2017). The results of this study showed that antioxidant capacities of muscles of male and female *E. massavensis* and *P. clarkii* are higher than antioxidant capacities in the exoskeleton. Binsan *et al.*, (2008) showed that the components extracted from cephalothoraxes of shrimp are responsible for its radical scavenging properties and antioxidant activity. The extracts of this species contain natural antioxidant mainly phenolic compounds (Seymour *et al.,* 1996). Similar results were shown by Gharibi *et al.* (2014) who used different techniques for determination of bioactivity level in shrimp *Penaeus seisulcatus*

Polyphenols are among the most widespread class of secondary metabolites in nature. Most

polyphenols arise from a common origin: the amino acids phenylalanine or tyrosine (Castellano *et al.,* 2012). In the present study, phenolic compounds that extracted by methanol from muscles of male and female *E. massavensis* and *P. clarkii* were found to be higher than that from exoskeleton and higher than the phenolic compounds that extracted by water. Hamdi (2011) showed the presence of high level of non-essential amino acid tyrosine and essential amino acid phenylalanine of muscle and carapace extracts of both *P. clarkii* and *E. massavensis*.

In the present study, total phenolic and flavonoid concentrations that extracted by methanol showed better results than those extracted by water from muscles and exoskeleton of both *E. massavensis* and *P. clarkii* (male and female). Azwanida (2015) suggested that methanol could act as an efficient solvent to extract the bioactive structurally unique secondary metabolites such as alkaloids, flavonoids and polyphenols. Total flavonoid compounds that extracted by methanol from the exoskeleton of male and female *E. massavensis* and *P. clarkii* were found to be higher than those of muscles. These results are in agreement with the previous study by Yanar *et al.* (2004) and Yasui *et al*. (2011). Arumugasamy and Cyril (2017) also reported presence of phenols and flavonoids in the crude extract of *H. pugilinus* and suggested that the feeding habit, environment factors and associated microorganisms of mollusca may contribute to the synthesis of biologically potential secondary metabolites.

The occurrence of carotenoids in crustaceans is mainly due to the absorption of pigments from the diet (Castillo *et al.,* 1982; Davies, 1985). We found that total carotenoid in the exoskeleton of female *E. massavensis* is higher than female *P. clarkii* by 1.73 folds and in the exoskeleton of male *E. massavensis* is higher than male *P. clarkii* by 1.23 folds. Sachindra *et al.* (2005) studied the distribution of carotenoid in different body components of four species of shrimp, *Penaeus monodon, Penaeus indicus, Metapenaeus dobsonii* and *Parapenaeopsisn stylifera*. Their results showed that the highest total carotenoid contents were observed in the head and carapace of *Parapenaeopsisn stylifera*

Natural fat-soluble pigments as carotenoid are being increasingly used to treat and prevent a wide variety of lifestyle-related diseases such as cancer, cardiovascular and immune diseases (Hamdi, 2011; Zhou et al., 2017). The most relevant biological functions of carotenoids are linked to their antioxidant properties, which directly emerge from their molecular structure (Guerin *et al.,* 2003; McNulty *et al.,* 2007). These results on the same line with (Sindhua and Sherief, 2011) that proved astaxanthin extracted from shell waste of *Aristeus alcocki* possessed significant hydroxyl radical scavenging activity, lipid peroxidation-inhibiting activities and superoxide radical-scavenging activity. However, we found that, carotenoid showed relatively weak antitumor activities on Ehrlich cells of both female and male *E. massavensis* and *P. clarkii.*

The results obtained showed that the carotenoid which extracted from the exoskeleton of marine *E. massiveness* has the highest antimicrobial effect. It has the strongest effect on Gram-positive bacteria (*E. coli*) and Gram-negative bacteria (*S. aureus*) as compared with Ampicillin and the strongest antifungal (*C. albicans*) activity as compared to Fluconazole. Similar results were obtained in previous studies which indicated that astaxanthin extracted from waste shrimp from Cochin, Kerala is effective against gram positive and gram negative bacteria, when compared with standard chloramphenicol (Ushakumari and Ramanujan, 2013). Bennedsen *et al.* (1999) reported that dietary astaxanthin was found to help fight symptoms of ulcer disease from Gram-negative bacteria. The present study showed that carotenoid that extracted from exoskeleton of female *E. massiveness* and *P. clarkii* showed high antitumor activity against Ehrlich Ascites Carcinoma (EAC) cells than that extracted from the male. These results are in agreement with previous studies reported that astaxanthin inhibited murine mammary tumour cell proliferation by up to 40%, in a dose-dependent fashion, when included in the culture medium (Kim *et al.,* 2001).

CONCLUSION

The results of this study can conclude that extracts of muscle and exoskeleton from both *E. massavensis* and *P. clarkii* have bioactive compounds such as carotenoids with antioxidant and antimicrobial activities. In addition, this study recommends using natural products as nutrients and/or supplements to protect consumers against free radicals and microbial infection.

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