Pharmacogn. Mag.
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ORIGINAL ARTICLE

Identification and Quantification of Fucoxanthin in Selected Carotenoid-Producing Marine Microalgae and Evaluation for their Chemotherapeutic Potential

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Submitted: 07-02-2019 Revised: 06-03-2019 Published: 26-08-2019

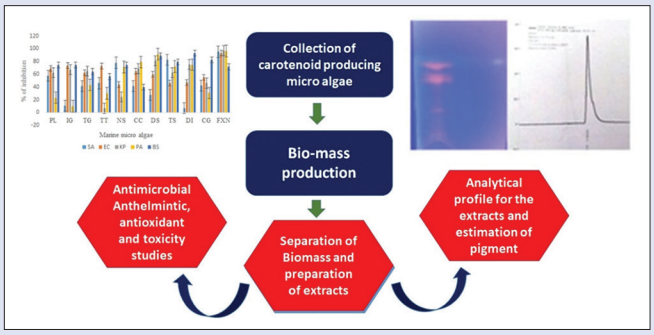
ABSTRACT

Background: Although there are about 18,500 compounds have been isolated and reported from marine resources, the prominence of drug discovery research on marine microalgae is still very less while comparing to other natural resources. Hence, this investigation was designed, especially on some carotenoid-producing marine microalgae to evaluate their chemotherapeutic efficacies including antibacterial, antifungal, antioxidant, hemolytic, and anthelmintic activities. **Objective:** The objective of this research is to evaluate the suitability of the selected marine microalgae for biological activities and to perform the identification and quantification of fucoxanthin in their methanol extracts using high-performance liquid chromatography (HPLC)–diode-array detector technique. **Materials and Methods:** The methanolic extracts of all 10 marine microalgae were screened for antibacterial, antifungal, antioxidant, hemolytic, and anthelmintic activities. The fucoxanthin was identified and quantified by thin-layer chromatography and HPLC techniques, respectively. **Results:** Among the test microalgae, *Isochrysis galbana* (IG) showed the presence of the highest concentration of fucoxanthin (5.93 mg/g dry weight) and also exhibited notable antioxidant activities (86%) at 80 mg/mL. In antimicrobial activities, *Dunaliella salina* (DS) demonstrated the promising antimicrobial activities (minimum inhibitory concentration [MIC]: 40 mg/mL) against Gram-negative bacteria and fungi while *Thalassiosira* species showed activity (MIC: 40 mg/mL) against *Staphylococcus aureus* and fungi. It was also noted that all test extracts were resistant to *Escherichia coli*. In anthelmintic activity against *Pheretima posthuma*, there are two microalgae, namely IG and *Chaetoceros gracilis,* exhibited considerable anthelmintic potential (with $P < 0.01$). **Conclusion:** From this study, it was concluded that DS and IG could serve as a promising source for further investigation to discover new antimicrobial leads and also demonstrated the positive correlation with the carotenoid content.

Key words: Chemotherapeutic, fucoxanthin, methanol, microalgae, screening

SUMMARY

- Ten different marine microalgae were cultured using f/2-Si medium for biomass production
- From the methanolic extracts, fucoxanthin was identified and detected by thin-layer chromatography and quantified by reversed-phase high-performance liquid chromatography techniques
- The methanolic extract concentrations of 20, 40, and 80 mg/mL were used to investigate the chemotherapeutic potential against antimicrobial, antioxidant, hemolytic, and anthelmintic activities
- Based on this study, *Isochrysis galbana* showed promising results for further investigation; in addition, *Chaetoceros gracilis* exhibited considerable anthelmintic activity ($P < 0.01$)
- The above mentioned two algae were also proven to produce a large amount of fucoxanthin while comparing with other test algae.



Abbreviations used: TLC: Thin-layer chromatography; HPLC: High-performance liquid chromatography; PDA: Photodiode array; UV: Ultraviolet; SPE: Solid-phase extraction; MIC: Minimum inhibitory concentration; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl; MPT: Mean paralysis time; MDT: Mean death time; PL: *Pavlova lutheri*; IG: *Isochrysis galbana*; TG: *Tetraselmis gracilis*; TT: *Tetraselmis tetrathele*; NS: *Navicula* sp.; CC: *Chaetoceros calcitrans*; DS: *Dunaliella salina*; TS: *Thalassiosira* sp.; DI: *Dicrateria inornata*; CG: *Chaetoceros gracilis*; SA: *Staphylococcus aureus* MTCC 727; EC: *Escherichia coli* MTCC 443; KP: *Klebsiella pneumoniae* MTCC 109; PA: *Pseudomonas aeruginosa* MTCC 424; BS: *Bacillus subtilis* MTCC 121; AB: *Aspergillus brasiliensis* MTCC 1344; AF: *Aspergillus fumigatus* MTCC 343; CA: *Candida albicans* MTCC 227.

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DOI: 10.4103/pm.pm_64_19

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INTRODUCTION

The present era is the renaissance period to undertake natural product research for identifying new drug molecules. The meta-analysis of reports available on natural product research revealed the significant societal impact of marine ecosystem as a source for discovery of new therapeutic drugs.[1] Ocean harbors inestimable number of organisms that could produce promising biological active substance due to their

Cite this article as: Peraman M, Nachimuthu S. Identification and quantification of fucoxanthin in selected carotenoid-producing marine microalgae and evaluation for their chemotherapeutic potential. Phcog Mag 2019;15:S243-9.

unique biosynthetic pathway for producing secondary metabolites.[2,3] So far, there are 18,500 compounds have been reported from marine resources, approximately about 90% of the compounds neither characterized nor detailed for therapeutic application.[4] Among the marine resources, the microalgae are still untapped resources to date, and the drug discovery research on microalgae is not completely explored.[5] There are hindering factors in microalgal research, and they are (a) difficulties in the collection and authentication process, as it requires molecular characterization including gene sequencing/amplification by polymerase chain reaction; (b) difficulties in the process optimization for production of microalgal culture at large scale, as it is affected by light, temperature, pH, salinity, and other specific nutritional or/and stress conditions; (c) structural diversity in the chemical nature of secondary metabolite production; (d) the percentage level of metabolite substance in the biomass of microalgae is very low; and (e) only little quantitative information is available on the algal rate as a source for bioactive molecule compared to plants, micro-organisms, and animals.[6,7] Despite these above-mentioned factors, microalgae are well known for its advantage of metabolite plasticity, and it means that the production of secondary metabolite can be enhanced by external stress such as nitrogen, oxygen, carbon dioxide, light, temperature, pH, and nutritional depletion. This has led a way to scientists for exploiting microalgae for the production of large number of novel compounds suitable for both health care and cosmetics.[8]

In the light of literature review, it is evident that microalgae can produce group of novel bioactive compound with pharmacological and other biological activities. Several of these bioactive compounds are now being more novel bioactive compounds screened and examined in the field of therapeutic application.[9] Sathasivam *et al.*[10] have summarized about 10 different microalgal carotenoids along with their potential use. The literature reported biological activities for the microalgal carotenoids are anti-inflammatory (astaxanthin and violaxanthin), antioxidant property (astaxanthin, β-carotene, fucoxanthin, canthaxanthin, and lutein), anti-cataracts (canthaxanthin, lutein, and zeaxanthin), anticancer (astaxanthin, β-carotene, and lutein), anti-obesity (fucoxanthin), and cardioprotective (beta-carotene and zeaxanthin). The search for the reports on antimicrobial activity of microalgal culture, it was found to be very little including phyta of dinophyta, heterokontophyta, haptophyta, and chlorophyta. In addition, there are few reports on the use of microalgal cell-free extracts as preservatives in food and feed formulations used for animals.[11] Fucoxanthin is one of the valuable bioactive pigments from vast group of carotenoid with various biological activities, but its use has very few applications due to low yield, poor extraction efficacy, and the difficulty in chemical synthesis. With these scopes, this present study was focused to screen fucoxanthin-producing marine microalgae for chemotherapeutic application. The emergence of antimicrobial resistance has witnessed the increasing mortality among bacteria-infected patients, due to multiple drug resistance nature of pathogenic microbes to the existing antibiotics.[12] This has motivated the scientists to drive their research attempts toward the discovery of new antibiotic molecule with novel mechanism of action. In search of new antimicrobial agents, molecules from natural origin are relatively more efficacious than synthetic agents due to their diversity in chemical structure, distribution, mechanism of action, and low toxicity profile.[13] The outlook on the outcome of natural product research has suggested that the marine microalgae could be chosen as resources for the discovery of newer chemotherapeutic agents. Pratt *et al.*[14] have isolated the first antimicrobial compound "chlorellin" from *Chlorella* species *w*hich demonstrated considerable activity against Gram-positive and Gram-negative bacteria. In subsequent years, few more antibacterial compounds have been reported, and they are eicosapentaenoic acid (*Phaeodactylum tricornutum*),[15] halogenated sesquiterpenes (*Laurencia dendroidea*),[16] sterols (*Gracilaria salicornia* and *Hypnea flagelliformis*),[17] unsaturated and saturated long-chain fatty acids (*Skeletonema costatum*),[18] and alpha-linolenic acids (*Chlorococcum HS-101*).[19] Similarly, there are few antifungal compounds such as karatungiols (*Amphidinium* sp.)[20] goniodomin A (*Goniodoma pseudogoniaulax),*[21,22] Gambieric acids A and B (*Gambierdiscus toxicus),*[23] and butanoic acid and methyl lactate (*Haematococcus pluvialis).*[24] In general, the antimicrobial potency of microalgae is due to its metabolites belonging to the chemical classes including terpene, indoles, acetogenins, phenols, fatty acids, and volatile halogenated compounds.[25]Among them, the antimicrobial activity of microalgae was well explained by the presence of terpenes and carotenoids such as alpha/beta-ionone, beta-cyclocitral, neophytadiene, and phytol.[26] Especially, the β-ionones and cyclic carotenoids interfere with respiratory chain of micro-organism and inhibit the microbial oxygen consumption.[27]

Considering the importance of carotenoid-related compounds for their interfering mechanism on respiratory chain of cell and their reliability for killing virulent microbes, the present work is designed to investigate some selected carotenoids producing microalgae for their chemotherapeutic potential against human pathogenic bacteria, fungi, and helminths. Here, we have selected ten microalgae and investigated them against Gram-positive bacteria (*Bacillus subtilis* MTCC 121) and Gram-negative bacteria (*Staphylococcus aureus* [SA] MTCC 727, *Escherichia coli* MTCC 443, *Klebsiella pneumoniae* MTCC 109, and *Pseudomonas aeruginosa* MTCC 424), fungi (*Aspergillus brasiliensis* MTCC 1344, *Aspergillus fumigatus* (AF) MTCC 343, and *Candida albicans* MTCC 227), and *Pheretima posthuma* (*Annelida*; Indian earthworm). Due to the free radical scavenging nature of carotenoids, this work was also extended to prove the antioxidant potential and hemolytic activity (for toxicity) for the methanol extracts of marine microalgae. Furthermore, we performed the identification of pigments by thin-layer chromatography (TLC) (on silica gel GF_{254}) and estimated the amount of fucoxanthin using high-performance liquid chromatographic (HPLC) technique.

MATERIALS AND METHODS

Microalgal strains and culture

A total of ten microalgal species were evaluated in this study. Among them, nine marine microalgae, namely *Pavlova lutheri* (PL), *Isochrysis galbana* (IG), *Tetraselmis gracilis* (TG), *Tetraselmis trahele* (TT), *Navicula* sp. (NS), *Chaetoceros calcitrans* (CC), *Dunaliella salina* (DS), *Thalassiosira* sp. (TS), and *Dicrateria inornata* (DI), were authenticated and purchased from Central Marine Fisheries Research Institute, Ernakulam, Kochi - 682 018 (with Voucher No. 10169). All algal isolates were collected in the month of June 2018. The authentication of marine algal *Chaetoceros gracilis* (CG) was supported by Virtis Bio Labs Pvt Ltd, Salem, India (Ref. No.: VBL/QO/001/2018-19) where the isolation was performed on f/2-Si medium. Chemicals and reagents used were procured from Hi-Media, Bombay, India. Laminar air flow chamber (Kleanzone Pvt. Ltd., Chennai) and Remi centrifuge (Remi R-8C) was used for culturing and handling of human pathogens. The wet biomass of algae was freeze-dried using freeze dryer (Lark Innovative Pvt. Ltd., Chennai).

Agilent LC-1200 HPLC system equipped with EZ chrome elite software, C_{18} Qualisil Gold Column (250 mm × 4.6 mm, 5 microns) photodiode array (PDA) detector, binary reciprocating pump, and manual Rheodyne injector (20 µL) was used in the identification and quantification of fucoxanthin in the methanolic extracts of microalgae. Thermo Fisher Scientific in microplate reader was used to estimate the fluorescence intensity of both standard and test samples. The absorbance value for the

determination percentage growth inhibition of microbes was measured using ultraviolet (UV)-visible spectrophotometer (Shimadzu, UV-1800, Japan).

Production of algal biomass

All the above authenticated algal cultures were re-inoculated in f/2-Si media using a stock culture (300–600 cells/mL). These cultures were scaled from 50 mL to 1 L using seawater enriched with f/2-Si medium.[28] Each flask containing respective microalgae was incubated at 22°C ± 2°C for 16 days. For algal biomass production, ratio of light and darkness cycle was 14: 10h at 60-65 μE/sm^2 light intensity. Once cultures grew, the entire algal biomass was harvested by centrifugation procedure at 4000 ×g. Then, the obtained biomass was rinsed twice with double distilled water to remove media components. Thus, the obtained algal biomass was lyophilized at −70°C for 48 h and stored at 4°C until further studies. The percentage (%) yield of algal biomass and the features are presented in Table 1.

Extract preparation

The extraction was performed based on the reported method.[29] The maceration techniques were employed for the preparation of algal extracts in which methanol was used as a solvent. The grown algal samples were filtered thrice using Whatman No. 1 filter paper and washed with distilled water for about 4–5 times. These were air-dried in hot air oven. About 10 mg of freeze-dried algal samples was suspended in 10 ml of methanol and shaken overnight at about 100 rpm and filtered. The residues were re-extracted under the same conditions. The contents were stored for a period of 5 days at room temperature (25°C ± 2°C) and then filtered using Whatman filter (125 mm). Further, the solvent was removed under vacuum to produce the dry crude extract.

In vitro antibacterial and antifungal activities

For antimicrobial activity, *Staphylococcus aureus* MTCC 727, *Escherichia coli* MTCC 443, *Klebsiella pneumoniae* MTCC 109, *Bacillus subtilis* MTCC 121, and *Pseudomonas aeruginosa* MTCC 424), fungi (*Aspergillus brasiliensis* MTCC 1344, *Aspergillus fumigatus* MTCC 343, and *Candida albicans* MTCC 227) were used. All bacterial and fungal pathogens were purchased from IMTECH (The Institute of Microbial Technology), Chandigarh - 160036, India. The extracts were screened in serial concentrations of 5, 10, 20, 40, and 80 mg/mL. For bacterial and fungal pathogens, amoxicillin and fluconazole were used as positive control at 1 μg/mL concentration. The dimethyl sulfoxide was used as a solvent (blank). The screening was performed as per the literature.[30] The media used for bacteria and fungi were Mueller-Hinton broth and potato dextrose broth, respectively. The percentage growth inhibition was determined using absorbance value obtained at 600 nm. The percentage inhibition data were obtained for each well using the absorbance of blank (broth) and positive control. The absorbance for percentage growth inhibition of extracts on fungi was measured at 530 nm using absorbance (OD_{530}) after the addition of 0.001% resazurin dye and incubation at 35°C for additional 2 h. The growth inhibition percentage of 90% was considered as minimum inhibitory concentration (MIC).

Table 1: Characteristics of microalgal biomass and its methanolic extract

Microalgae	Color of biomass	Biomass production (g/L, DW)	Appearance of extract	Number of compounds in TLC[a]	Presence of fucoxanthin (R_f: 0.85)	Amount of fucoxanthin by HPLC[b] (mg/g DW)
PL	Greenish brown	3.32	Pale green	3	Yes	0.40
IG	Brown green	1.73	Dark green	4	Yes	5.93
TG	Green	1.45	Blackish green	4	No	-
TT	Green brown	2.21	Pale yellow	3	No	-
NS	Dark brown	1.42	Green	5	Yes	0.73
CC	Greenish brown	1.95	Yellowish green	8	Yes	0.36
DS	Green	2.43	Yellowish green	5	Yes	1.23
TS	Brown	2.21	Pale yellow	7	Yes	0.56
DI	Brown	1.87	Pale yellow	5	No	-
CG	Greenish brown	0.55	Pale green	4	Yes	1.92

[a]TLC profile - mobile phase: n-hexane and ethyl acetate at the ratio of 60:40% *v/v*; TLC plate: Precoated silica gel GF_{254}; Detection: UV chamber; Spot: Spherical and manual. [b]HPLC - separated on C_{18} column and quantified using fucoxanthin standard (t_R: 3.7±0.1 min). PL: *Pavlova lutheri*; IG: *Isochrysis galbana*; TG: *Tetraselmis gracilis*; TT: *Tetraselmis trahele*; NS: *Navicula* sp.; CC: *Chaetoceros calcitrans*; DS: *Dunaliella salina*; TS: *Thalassiosira* sp.; DI: *Dicrateria inornata*; CG: *Chaetoceros gracilis*; TLC: Thin-layer chromatography; HPLC: High-performance liquid chromatographic; DW: Dry weight

Table 2: Antioxidant and hemolytic activities of methanolic extract of microalgal biomass (*n*=3)

Extract code	Antioxidant activities by ABTS (percentage of inhibition)			Antioxidant activity by DPPH (percentage of inhibition)			Hemolytic activity (percentage of lysis)		
	20 mg/mL	40 mg/mL	80 mg/mL	20 mg/mL	40 mg/mL	80 mg/mL	20 mg/mL	40 mg/mL	80 mg/mL
PL	14.32±0.23	36.19±0.39	56.43±0.62	41.65±0.43	52.45±0.68	81.49±0.89	<5	<5	5.41±0.12
IG	23.36±0.34	41.30±0.44	62.93±0.72	50.23±0.59	64.78±0.79	86.89±0.93	<5	<5	9.24±0.19
TG	10.78±0.19	26.30±0.32	48.19±0.59	31.93±0.38	45.49±0.57	69.27±0.73	<5	6.08±0.43	11.18±0.32
TT	12.43±0.24	33.94±0.41	52.56±0.57	35.89±0.44	49.23±0.62	74.26±0.79	<5	7.03±0.31	12.56±0.48
NS	11.56±0.17	29.01±0.37	51.99±0.64	33.56±0.41	47.89±0.74	71.99±-0.75	<5	<5	5.48±0.17
CC	13.29±0.21	32.57±0.45	56.89±0.67	49.10±0.61	53.01±0.71	83.53±0.92	<5	<5	9.11±0.12
DS	15.34±0.29	34.92±0.46	49.86±0.61	39.23±0.56	58.10±0.82	79.39±0.82	<5	<5	8.52±0.47
TS	12.89±0.22	31.90±0.37	53.27±0.54	36.54±0.49	48.89±0.60	80.97±0.85	<5	<5	7.77±0.24
DI	12.14±0.16	30.45±0.35	51.62±0.63	34.29±0.42	47.01±0.59	78.19±0.80	<5	9.08±0.43	12.61±0.28
CG	11.24±0.18	28.12±0.32	52.72±0.69	34.85±0.46	46.55±0.56	72.63±0.77	<5	<5	9.02±0.23
Fucoxanthin	45.89±0.49	73.72±0.84	81.29±0.82	78.21±0.79	83.58±0.84	86.12±0.91	<5	<5	7.39±0.41

PL: *Pavlova lutheri*; IG: *Isochrysis galbana*; TG: *Tetraselmis gracilis*; TT: *Tetraselmis trahele*; NS: *Navicula* sp.; CC: *Chaetoceros calcitrans*; DS: *Dunaliella salina*; TS: *Thalassiosira* sp.; DI: *Dicrateria inornata*; CG: *Chaetoceros gracilis*; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl

In vitro antioxidant activity

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay was measured based on the method described by Foo *et al.*[31] For control, 1 mL of each 7-mM ABTS aqueous solution and 2.4 mM of ammonium per sulfate were added and incubated in dark for 14 h at room temperature (25°C ± 2°C). The ABTS solution was diluted with ethanol until the absorbance of 0.70 was read at 734 nm (control). For test, 900 µL of the prepared ABTS reagent was added to the 100-µL methanolic extract of test algae and then vortexed and incubated for 6 min at room temperature (25°C ± 2°C). The absorbance value was measured at 734 nm. The ABTS scavenging activity is measured as antioxidant potential of the test compounds. Test sample concentration ranges from 20, 40, to 80 mg/mL.

$$\text{ABTS scavengingactivity} = \frac{\text{control} - \text{test}}{\text{control}} \times 100$$

2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay was measured based on method described by Brand *et al.*[32] For test, methanolic extract was made into the concentrations of 20, 40, and 80 mg/mL. To the above test concentrations, 3 mL of methanolic solution of DPPH (100 mM) was added and incubated for 30 min in dark. Optical density was measured at 517 nm. The solution consists of methanol, and DPPH reagent was used as control whereas methanol was served as blank.

$$\%\text{inhibition} = \frac{\text{control} - \text{test}}{\text{control}} \times 100$$

In vitro hemolytic activity

The toxicity of the test extracts was tested by *in vitro* hemolytic activity previously reported by Merlino *et al.*[33] and Mangoni *et al.*[34] About 5 mL of fresh blood was centrifuged at 4000 rpm to separate erythrocytes. The obtained erythrocytes were suspended in phosphate-buffered saline (pH 7.4) and it was diluted to the cell count of 6.4×10^8 cells/mL. Stock solutions of 50 and 100 mg/mL were prepared for all test extracts using saline. The stock solution of extracts (250 µL) and 100 µL of a previously prepared red blood cell (RBC) suspension were added in Eppendorf tubes. The saline was used as negative control while 20% w/v of Triton X (20 µL) was served as positive control. All tubes were incubated at 37°C ± 2°C for 40 min and then centrifuged at 4000 rpm for 15 min. The obtained absorbance at 404 nm was used to calculate percentage lysis.

$$\text{Percentage lysis} = ([A_{\text{test}} - A_{\text{negative control}}]/[A_{\text{positive control}} - A_{\text{blank}}]) \times 100$$

In vitro antihelminthic activity

The Indian earthworms, *P. posthuma* (*Annelida*) with an average size of 6–9 cm, were collected from waterlogged soil and washed thrice with potable water to remove soil and adhered dirt. The *in vitro* anthelminthic screening for all the ten algal extracts was done as per the reported procedure.[35] Three worms (n = 3) were used in the testing of each extract. The Petri dish was used to hold the suspended earthworms in media consisting of test extracts (20, 40, and 80 mg/mL) and standard compound (albendazole at 1, 5, and 10 mg/mL). The mean paralysis time (MPT) was noted when there was no movement of earthworm without external shaking of media. However, the mean death time (MDT) was recorded only when there was no movement even after socking in the warm water (50°C).

Detection and quantification of pigments

Identification of pigments by thin-layer chromatography

A mobile phase consisting of n-hexane and ethyl acetate at 60:40% v/v was used to run the test extracts on silica gel GF_{254}-coated aluminum plate. The R_f value of standard (fucoxanthin) is used to identify the presence of fucoxanthin in methanol extract of microalgae.

Quantification of fucoxanthin in methanol extract by high-performance liquid chromatography

Agilent LC-1200 HPLC system equipped with EZ chrome elite software, C_{18} Qualisil Gold Column (250 mm × 4.6 mm, 5 microns) PDA detector, binary reciprocating pump, and manual Rheodyne injector (20 µL) was used in the identification and quantification of fucoxanthin in the methanolic extracts of microalgae. The test extracts and standard (1–10 µg/mL) were prepared in HPLC grade methanol. The solid-phase extraction (SPE) was adapted to purify the methanolic extract, and the elution was done with HPLC grade acetonitrile. The separation of both sample and standard was carried out on C_{18} Qualisil Gold (250 mm × 4.6 mm, 5 µ) column using a mixture of mobile phase consisting of acetonitrile and water (pH 3.0; adjusted using 10% orthophosphoric acid). The flow rate was 1 mL/min, the detection was by PDA @ 264 nm, and the peak purity was assessed by purity plot (>0.999). Under the above condition, purified samples from SPE and standard were injected into the column in triplicate. The peak height/peak area was used to quantify the fucoxanthin concentration in the methanol extracts of microalgae.

Statistical analysis

All data were determined from three/two independent experiments. Mean values and standard deviations (SDs) were calculated with Microsoft Excel software, and all the data are expressed as the mean ± SD. The P value was used for expressing anthelmintic activities.

RESULTS

Culture collection and biomass extraction

To investigate the chemotherapeutic potential of carotenoid-producing microalgae, ten different microalgae, namely PL, IG, TG, TT, NS, CC, DS, TS, DI, and CG, were selected. The algal biomass of each sample was subjected to cold extraction using methanol as per the procedure described by Foo *et al.*[31] Table 1 shows the characteristics of marine algal biomass and their methanolic extracts along with TLC profile and fucoxanthin content by HPLC method. Figure 1 shows the structure of fucoxanthin.

In vitro antimicrobial activities

Methanolic extracts of all ten micro algae were screened against all bacterial and fungal human pathogens at the concentration 20, 40, and 80 mg/mL. The percentage inhibition was obtained and then MIC was reported [Table 3]. The study was performed based on the microdilution assay. The results are interpreted as a bar graph and presented in Figures 2 and 3.

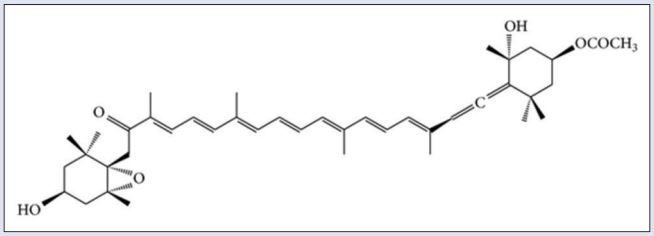

Figure 1: Structure of fucoxanthin

Table 3: Antimicrobial activity data of methanolic extract (at 40 mg/mL) of microalgal biomass (*n*=3)

Extract code	SA		EC		KP		PA		BS		AB		AF		CA	
	Percentage[a]	MIC[b]	Percentage	MIC	Percentage	MIC	Percentage	MIC	Percentage	MIC	Percentage	MIC	Percentage	MIC	Percentage	MIC
PL	57.47	80	68.80	80	62.16	80	23.07	>80	74.63	80	74.63	80	76.71	80	70.25	80
IG	10.95	>80	73.60	80	67.56	80	10.09	>80	74.4	80	74.40	80	84.93	40	23.58	>80
TG	41.37	80	62.40	80	64.86	80	43.35	>80	64.4	80	64.40	80	83.56	40	69.23	80
TT	45.97	80	72.80	80	06.40	>80	30.06	>80	56.4	80	56.40	80	57.53	80	10.25	>80
NS	78.16	80	44.00	>80	24.32	>80	71.32	80	74.4	>80	74.40	80	36.98	>80	87.17	40
CC	41.37	>80	64.80	80	68.91	80	79.02	80	40	80	40.00	>80	31.50	>80	49.35	>80
DS	27.58	>80	60.00	80	81.08	40	91.60	40	89.26	80	89.26	40	87.67	40	81.02	40
TS	82.75	40	46.40	80	62.16	80	72.02	80	79.51	40	79.51	40	78.08	40	68.20	80
DI	06.47	>80	47.20	80	75.67	40	74.82	80	93.17	80	93.17	40	76.71	80	69.23	80
CG	42.52	80	55.20	80	45.94	>80	30.76	>80	82.92	80	82.92	40	78.08	40	65.12	80
Blank	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Positive control	97.2	≤0.1	95.5	≤3.5	98.3	≤4.3	97.1	≤4.0	92.3	≤0.2	93.6	≥0.2	96.1	≥0.1	93.4	≥0.1
Fucoxanthin	95.2	1	92.8	2	97.1	1	96.4	1	71.78	4	94.7	2	95.6	1	91.4	2

[a]Percentage of growth inhibition, [b]MIC in 40 mg/mL; For bacterial pathogens, amoxicillin and, for fungal pathogens, fluconazole were used as positive control (at 1 µg/mL concentration); Fucoxanthin (at 1 mg/mL concentration). PL: *Pavlova lutheri*; IG: *Isochrysis galbana*; TG: *Tetraselmis gracilis*; TT: *Tetraselmis trahele*; NS: *Navicula* sp.; CC: *Chaetoceros calcitrans*; DS: *Dunaliella salina*; TS: *Thalassiosira* sp.; DI: *Dicrateria inornata*; CG: *Chaetoceros gracilis*; SA: *Staphylococcus aureus* MTCC 727; EC: *Escherichia coli* MTCC 443; KP: *Klebsiella pneumoniae* MTCC 109; PA: *Pseudomonas aeruginosa* MTCC 424; BS: *Bacillus subtilis* MTCC 121; AB: *Aspergillus brasiliensis* MTCC 1344; AF: *Aspergillus fumigatus* MTCC 343; CA: *Candida albicans* MTCC 227

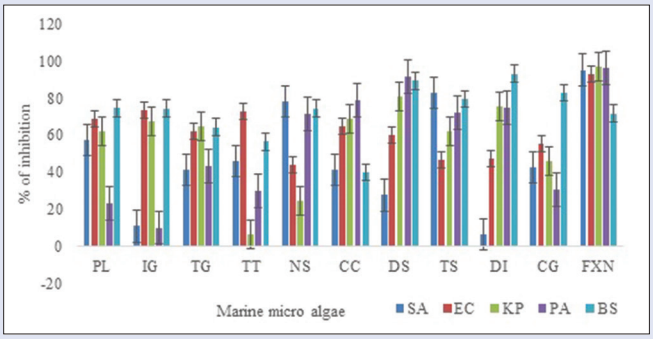

Figure 2: Antibacterial activity of microalgal extracts in percentage growth inhibition (at 40 mg/mL)

In vitro antioxidant and hemolytic activities

Table 2 shows antioxidant and hemolytic activities of ten micro algae. For antioxidant assay, both ABTS scavenging and DPPH scavenging activities were performed and results were expressed in percentage of inhibition. Similarly, for hemolytic activity of methanolic extracts was performed on RBC and percentage lysis was calculated based on spectroscopic absorption value and expressed in percentage of lysis for all the ten marine algae.

In vitro anthelmintic activity

For anthelmintic activity, *P. posthuma* (test earthworms) were used. The results are shown in Table 4, obtained as MPT and MDT, and were expressed at concentrations of 20, 40, and 80 mg/mL.

Identification and quantification of fucoxanthin

The fucoxanthin in the methanolic extract of test marine algae was identified by TLC on silica gel GF_{254} using the mobile phase consisting of n-hexane and ethyl acetate (60:40% v/v). The compounds are visualized under daylight and UV light and then reported. RP-HPLC method was used to determine amount of fucoxanthin in selected micro algae. Based on the TLC, there were seven algal extracts which were subjected for purification by SPE and analyzed using fucoxanthin as standard. Table 1 shows that the HPLC analysis revealed the presence fucoxanthin in methanolic extracts of marine algae.

DISCUSSION

The microalgae investigated were PL, IG, TG, TT, NS, CC, DS, TS, DI, and CG [Table 1]. Among the test microalgae, algal extract of IG showed the high percentage (%) of fucoxanthin about 5.93 mg/g dry weight (DW), whereas CC extract showed the least percentage of fucoxanthin with 0.36 mg/g DW. The biomass production in g/L (DW) was found to be in the range from 3.32 (PL) to 0.55 (CG). Foo *et al.*[31] described about fucoxanthin profile of both marine microalga (CC) and macroalga (*Saccharina japonica*) by comparing standard fucoxanthin. The methanolic extracts of all the microalgae were appeared as pale/dark green or yellowish green, whereas the color of biomass was light greenish brown or brown or green, as shown in Table 1.

The antimicrobial activity of methanolic extract of microalgae was screened against five different bacterial and three different fungal pathogens. For antimicrobial test, microdilution assay was performed and the percentage of growth inhibition was measured using microplate reader. The percentage inhibition of 90 was considered to find the MIC. Table 3 shows the MIC of extracts against each test pathogen whereas

Table 4: Anthelmintic activity of methanolic extract of microalgal biomass against *Pheretima posthuma*

Extract code	Mean paralysis time (n=3; in min)			Mean death time (n=3; in min)		
	20 mg/mL	40 mg/mL	80 mg/mL	20 mg/mL	40 mg/mL	80 mg/mL
PL	84±3.4*	43±1.2*	35±0.4*	98±4.5*	63±1.4*	47±0.4*
IG	44±5.3**	32±2.2**	22±1.2**	51±5.3**	43±2.2**	28±1.2**
TG	63±5.4*	46±1.5*	32±0.6*	80±5.4*	56±1.6*	43±0.6
TT	75±1.5*	62±1.3*	51±0.4*	87±1.5*	68±1.3*	59±0.4*
NS	43±1.6*	33±1.8*	20±1.1*	58±1.6*	41±1.8*	32±1.1*
CC	43±1.4*	34±1.6*	26±0.4*	57±1.4*	45±1.5*	36±0.9*
DS	49±2.3*	34±1.2*	22±1.8*	53±2.3*	44±1.2*	36±1.7*
TS	58±5.6*	38±4.8*	29±1.2*	76±5.6*	58±4.8*	38±1.3*
DI	59±2.5*	42±2.2*	38±1.3*	75±2.5*	68±2.3*	52±1.4*
CG	41±5.2**	28±3.1**	22±1.6**	54±5.2**	39±3.1**	28±1.7**
Albendazole	35±1.3*	22±1.2*	17±1.9*	40±1.3*	31±1.5*	26±1.8*
Negative control	>160	>160	>160	>160	>160	>160

**P<0.01 compared to negative control; *P<0.05 compared to negative control. PL: *Pavlova lutheri*; IG: *Isochrysis galbana*; TG: *Tetraselmis gracilis*; TT: *Tetraselmis trahele*; NS: *Navicula* sp.; CC: *Chaetoceros calcitrans*; DS: *Dunaliella salina*; TS: *Thalassiosira* sp.; DI: *Dicrateria inornata*; CG: *Chaetoceros gracilis*

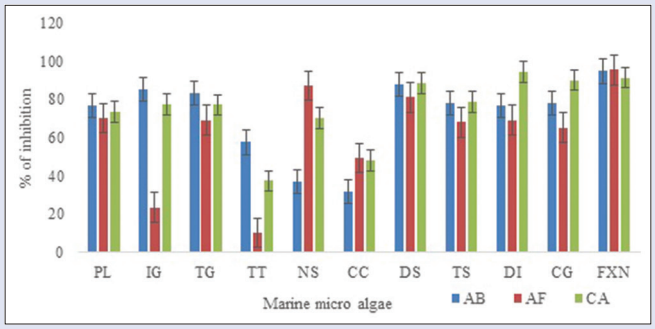

Figure 3: Antifungal activity of microalgal extracts in percentage growth inhibition (at 40 mg/mL)

Figures 1 and 2 show the percentage of growth inhibition of extracts at concentration of 40 mg/mL against test pathogens.

Among all the ten microalgae, DS exhibited the promising activity (MIC: 40 mg/mL) against Gram-negative bacteria and fungi [Table 3]. The extract TS showed activity against SA with 40 mg/mL. Similarly, TG showed activity against AF at 40 mg/mL. The extract CG showed predominant antifungal activity. The extract of DS has activity against PA (MIC: 40 mg/mL). Notably, only two extracts (*Dunaliella salina* (DS) and *Dicrateria inornata* (DI)) were activity against KP at 40 mg/mL. In connection with fungal activities, most of the extract IG, TG, DS, TS, and CG showed MIC of 40 mg/mL against AF but not on *C. albicans*. However, most of the extracts demonstrated considered percentage inhibition about >80% at 80 mg/mL. Similarly, pressurized solvent extracts (hexane, petroleum ether, hexane, and water) of DS were screened for antimicrobial activity against *E. coli,* SA, *C. albicans,* and *Aspergillus niger.*[26]

In antioxidant activity assay for percentage of inhibition by ABTS scavenging activity, except TG (48%) and DS (49%), the remaining algal isolates show >50% of inhibition of methanolic extract at 80 mg/mL. Maximum percentage of inhibition (62%) was noticed with the extract of IG at 80 mg/mL. The algal isolate TG showed 10%, 26%, and 48% of inhibitions at concentrations of 20, 40, and 80 mg/mL, respectively. Similarly, in the percentage of inhibition by DPPH scavenging activity, except TG (69%), all other algal isolates show the percentage of inhibition in the range between 70% and 80% at a concentration of 80 mg/mL. The percentage inhibitions of IG were 50%, 64%, and 86% at concentrations of 20, 40, and 80 mg/mL, respectively. The previous study reported that total antioxidant activity (ABTS and DPPH) of crude methanolic extract of CC shows high activity when compared to other solvent systems such as diethyl ether-water, dichloromethane-water, and ethyl acetate-water.[31,36]

Similarly, percentage of lysis for hemolytic activity of all the ten algal extracts at concentration 20, 40, and 80 mg/mL is expressed in Table 2. Among ten marine algal isolates, isolate CC shows 6% and 11% of lysis at concentration of 40 and 80 mg/mL, respectively. This result indicates that the methanolic extract of all the ten marine microalgae shows less toxicity to RBCs and suitable for investigation of drugs for humans.

Table 4 shows that the anthelmintic activity of methanolic extract of microalgae was studied at 20, 40, and 80 mg/mL concentrations against *P. posthuma*. Based on data obtained from this study, *P* value was calculated. Among ten algal extracts, only CG at 40 and 80 mg/mL shows paralysis (MPT) of *P. posthuma* after 22 and 28 min, respectively. In MDT, only CG at 40 and 80 mg/mL showed mortality of *P. posthuma* after 39 and 28 min, respectively. Methanolic extracts (at 80 mg/mL concentration) of both IG and CG shows mortality after 28 min of incubation.

From this study, there is some correlation between fucoxanthin producer and nonproducers. In preliminary screening for fucoxanthin production, except TG, TT, and DI, the remaining all seven marine algae produce fucoxanthin, with respect to antimicrobial, antioxidant, hemolytic, and anthelmintic activities. The correlation coefficient between fucoxanthin content and antioxidant activity was found to be >0.6. This value indicated that the antioxidant property of the extract is contributed by fucoxanthin in methanolic extract of algal isolates.

Out of ten marine microalgae, algal isolate TT shows 77% of growth inhibition for *E. coli* MTCC 443 and DI for *A. brasiliensis* MTCC 1344 with 93% of inhibition.[26] Similarly, for antioxidant activity, DI shows 78% of inhibition in DPPH assay whereas TI and TG show only 74% and 69% of inhibition at 80 mg/mL concentration. In case of percentage of lysis for hemolytic activity, all the three isolates, i.e., TG, TT, and DI show that the highest percentage lysis ranges from 6% to 9.5% at 40 mg/mL and 11%–12% at 80 mg/mL concentrations. Out of 10 isolates, isolate DI shows the highest percentage of inhibition at 40 mg/mL (9%) and 80 mg/mL concentrations (12%). Similarly, TG, TT, and DI did not show MPT and MDT even after 30 min of incubation by increasing concentration from 20 mg/mL to 80 mg/mL concentration. TG alone shows paralysis (MPT) of *P. posthuma* after 32 min at 80 mg/mL concentration. It was confirmed that isolates have the highest fucoxanthin production potential (IG with percentage of fucoxanthin about 5.93 mg/g DW) that shows the highest percentage of growth inhibition of bacterial and fungal pathogens as well as highest percentage inhibition in antioxidant, hemolytic, and anthelmintic activities.

CONCLUSION

In summary, among all the ten marine algal extracts, IG produces a higher concentration of fucoxanthin (5.93 mg/mL DW). In overall, chemotherapeutic activity of methanolic extract of selected marine microalgal isolate, IG, showed the promising antioxidant activities with 86% of inhibition at 80 mg/mL. In antimicrobial activities, DS exhibited a promising activity (MIC: 40 mg/mL) against Gram-negative bacteria and fungi. In anthelmintic activity against *P. posthuma*, two microalgae, namely IG and CG, exhibited considerable anthelmintic activity ($P < 0.01$). From this study, it was concluded that DS and IG will serve as a promising source for further investigation and chemotherapeutic application which has a positive correlation with the carotenoid content as well.

Acknowledgements

The authors would like to thank the management of Kumaraguru College of Technology (an autonomous institute, affiliated to Anna University, Chennai) for providing research facility. The authors thank the RERDS-Centre for Pharmaceutical Research, Raghavendra Institute of Pharmaceutical Education and Research Campus, Anantapur, Andhra Pradesh, India, for their support in HPLC analysis and anthelmintic and hemolytic activities.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Molinski TF. All natural: The renaissance of natural products chemistry. J Org Chem 2014;79:6765.
2. Blunt JW, Carroll AR, Copp BR, Davis RA, Keyzers RA, Prinsep MR, *et al.* Marine natural products. Nat Prod Rep 2018;35:8-53.
3. Kiuru P, D'Auria MV, Muller CD, Tammela P, Vuorela H, Yli-Kauhaluoma J, *et al.* Exploring marine resources for bioactive compounds. Planta Med 2014;80:1234-46.
4. Olaizola M. Commercial development of microalgal biotechnology: From the test tube to the marketplace. Biomol Eng 2003;20:459-66.
5. Abida H, Ruchaud S, Rios L, Humeau A, Probert I, De Vargas C, *et al.* Bioprospecting marine plankton. Mar Drugs 2013;11:4594-611.
6. Borowitzka MA. Pharmacaeuticals and Agrochemicals from Microalgae. Chemicals from Microalgae. London: Taylor and Francis; 1999. p. 313-52.
7. Molinski TF, Dalisay DS, Lievens SL, Saludes JP. Drug development from marine natural products. Nat Rev Drug Discov 2009;8:69-85.
8. Guedes AC, Amaro HM, Malcata FX. Microalgae as sources of high added-value compounds – A brief review of recent work. Biotechnol Prog 2011;27:597-613.
9. Sadhasivam R, Pongpadung P, Praiborn J, Chirapart A, Trakulnaleamsai S, Roytrakyl S, *et al.* Optimizing NaCl and KNO_3 concentrations for high Beta-carotene production in photobioreactor by *Dunaliella salina* KU11 isolated from saline soil sample. Chiang Mai J Sci 2018;45:105-15.
10. Sathasivam R, Ki JS. A review of the biological activities of microalgal carotenoids and their potential use in healthcare and cosmetic industries. Mar Drugs 2018;16. pii: E26.
11. Tramper J, Battershill C, Brandenburg W, Burgess G, Hill R, Luiten E, *et al.* What to do in marine biotechnology? Biomol Eng 2003;20:467-71.
12. Peraman R, Varma RV, Reddy YP. Re-engineering nalidixic acid's chemical scaffold: A step towards the development of novel anti-tubercular and anti-bacterial leads for resistant pathogens. Bioorg Med Chem Lett 2015;25:4314-9.
13. Cheuka PM, Mayoka G, Mutai P, Chibale K. The role of natural products in drug discovery and development against neglected tropical diseases. Molecules 2016;22. pii: E58.
14. Pratt R, Daniels TC, Eiler JJ, Gunnison JB, Kumler WD, Oneto JF, *et al.* Chlorellin, an antibacterial substance from chlorella. Science 1944;99:351-2.
15. Desbois AP, Mearns-Spragg A, Smith VJ. A fatty acid from the diatom *Phaeodactylum tricornutum* is antibacterial against diverse bacteria including multi-resistant *Staphylococcus aureus* (MRSA). Mar Biotechnol (NY) 2009;11:45-52.
16. Biá Ventura TL, da Silva Machado FL, de Araujo MH, de Souza Gestinari LM, Kaiser CR, de Assis Esteves F, *et al.* Nitric oxide production inhibition and anti-mycobacterial activity of extracts and halogenated sesquiterpenes from the Brazilian red alga *Laurencia dendroidea* J. Agardh. Pharmacogn Mag 2015;11:S611-8.
17. Saeidnia S, Gohari AR, Shahverdi AR, Permeh P, Nasiri M, Mollazadeh K, *et al.* Biological activity of two red algae, *Gracilaria salicornia* and *Hypnea flagelliformis* from Persian Gulf. Phcog Res 2009;1:428-30.
18. Naviner M, Berge JP, Durand P, Le BH. Antibacterial activity of the marine diatom *Skeletonema costatum* against aquacultural pathogens. Aquaculture 1999;174:15-24.
19. Ohta S, Chang T, Ikegami N, Kondo M, Miyata H. Antibiotic substance produced by a newly isolated marine microalga, *Chlorococcum* HS-101. Bull Environ Contam Toxicol 1993;50:171-8.
20. Washida K, Koyama T, Yamada K, Kitab M, Uemura D. Karatungiols A and B, two novel antimicrobial polyol compounds, from the symbiotic marine dinoflagellate *Amphidinium* sp. Tetrahedron Lett 2006;47:2521-5.
21. Bhadury P, Wright PC. Exploitation of marine algae: Biogenic compounds for potential antifouling applications. Planta 2004;219:561-78.
22. Abe M, Inoue D, Matsunaga K, Ohizumi Y, Ueda H, Asano T, *et al.* Goniodomin A, an antifungal polyether macrolide, exhibits antiangiogenic activities via inhibition of actin reorganization in endothelial cells. J Cell Physiol 2002;190:109-16.
23. Nagai H, Mikami Y, Yazawa K, Gonoi T, Yasumoto T. Biological activities of novel polyether antifungals, gambieric acids A and B from a marine dinoflagellate *Gambierdiscus toxicus*. J Antibiot (Tokyo) 1993;46:520-2.
24. Santoyo S, Rodriguez MI, Cifuentes A, Jaime L, Garcia BR, Senorans FJ, *et al.* Green processes based on the extraction with pressurized fluids to obtain potent antimicrobials from *Haematococcus pluvialis* microalgae. LWT Food Sci Technol 2009;42:1213-8.
25. Cardozo KH, Guaratini T, Barros MP, Falcão VR, Tonon AP, Lopes NP, *et al.* Metabolites from algae with economical impact. Comp Biochem Physiol C Toxicol Pharmacol 2007;146:60-78.
26. Herrero M, Ibáñez E, Cifuentes A, Reglero G, Santoyo S. *Dunaliella salina* microalga pressurized liquid extracts as potential antimicrobials. J Food Prot 2006;69:2471-7.
27. Larroche C, Cruely C, Gros JB. Fed-batch biotransformation of β-ionone by *Aspergillus niger*. Appl Microbiol Biotechnol 1995;43:222-7.
28. Guillard RR, Ryther JH. Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt, and Detonula confervacea (cleve) gran. Can J Microbiol 1962;8:229-39.
29. Kim SM, Jung YJ, Kwon ON, Cha KH, Um BH, Chung D, *et al.* A potential commercial source of fucoxanthin extracted from the microalga *Phaeodactylum tricornutum*. Appl Biochem Biotechnol 2012;166:1843-55.
30. Peraman R, Tiwari AK, Vani M, Hemanth J, Sree YG, Karthik K, *et al.* New camphor hybrids: Lipophilic enhancement improves antimicrobial efficacy against drug-resistant pathogenic microbes and intestinal worms. Med Chem Res 2018;27:1728-39.
31. Foo SC, Fatimah MY, Maznah I, Mahiran B, Kim WC, Nicholas MH, *et al.* Production of fucoxanthin-rich fraction (FxRF) from a diatom, *Chaetoceros calcitrans* (Paulsen) Takano 1968. Algal Res 2015;12:26-32.
32. Brand WW, Cuvelier M, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT Food Sci Technol 1995;28:25-30.
33. Merlino F, Carotenuto A, Casciaro B, Martora F, Loffredo MR, Di Grazia A, *et al.* Glycine-replaced derivatives of [Pro3, DLeu9]TL, a temporin L analogue: Evaluation of antimicrobial, cytotoxic and hemolytic activities. Eur J Med Chem 2017;139:750-61.
34. Mangoni ML, Carotenuto A, Auriemma L, Saviello MR, Campiglia P, Gomez-Monterrey I, *et al.* Structure-activity relationship, conformational and biological studies of temporin L analogues. J Med Chem 2011;54:1298-307.
35. Liu GL, Hao B, Liu SP, Wang GX. Synthesis and anthelmintic activity of osthol analogs against *Dactylogyrus* intermedius in goldfish. Eur J Med Chem 2012;54:582-90.
36. Madhavan J, Chandrasekharan S, Priya MK, Godavarthi A. Modulatory effect of carotenoid supplement constituting lutein and zeaxanthin (10:1) on anti-oxidant enzymes and macular pigments level in rats. Pharmacogn Mag 2018;14:268-74.