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Research Article

ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF ETHYL ACETATE EXTRACT OF THE INDIAN GREEN MUSSEL *PERNA VIRIDIS*

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ABSTRACT

Objective: Invertebrates are the most explored groups in the field of cytotoxic/ anti-cancer marine natural products research, considering their ecological and evolutionary significance. Hence an attempt has been done to evaluate the possible antioxidant and cytotoxic activities of the Indian green mussel *Perna viridis.* **Methods**: Qualitative analysis of the secondary metabolites present in the ethyl acetate extract of *P. viridis* was done using general detection reagents and spray reagents on TLC. Antioxidant activity of the same was assessed by DPPH radical scavenging assay. Brine shrimp, Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) were employed for evaluating cytotoxicity of the extract. MTT assay of the extract was performed with two human cancer cell lines namely, HCT 116 and MCF 7. **Results:** General detection reagents and spray reagents showed the presence of different secondary metabolites in the ethyl acetate extract of *P. viridis*. The extract showed good scavenging activity against DPPH radical (IC_{50} 0.616 mg/ml). It also proved significantly cytotoxic towards brine shrimp, DLA and EAC cells. MTT assay on human cell lines proved to be more cytotoxic towards MCF 7 than for HCF 116 for the extract. **Conclusion**: The results shows that ethyl acetate extract of *P. viridis* possess both antioxidant and cytotoxic activities, which can be attributed to the presence of secondary metabolites like phenolics and alkaloids.

Keywords: *P. viridis,* antioxidant activity, DPPH, cytotoxic activity, DLA, EAC, MTT assay.

INTRODUCTION

Cancer is a leading cause of death worldwide and the figure is projected to be continuously rising, with an estimated 12 million in 2030. A major problem with present cancer chemotherapy is the serious deficiency of active drugs for the curative therapy of tumours. To satisfy the demand for new anti-cancer drugs, scientists are scouring the world for new species of plants, animals, marine and micro-organisms which demonstrate potent anti-cancer properties. Rainforests, deep sea thermal vents and coral reefs are examples of ecosystems which are currently being explored for novel sources of anti-neoplastic molecules [1, 2 & 3].

In recent years, more and more researchers have come to the realization that marine organisms hold immense potential as a source of novel molecules and new anticancer agents. Over the past decade intense efforts were made to more effectively explore new anticancer clinical and preclinical agents from the rich chemical diversity offered by marine life. The core reason behind the production of highly active compounds from marine organisms can be justified by the so called dilution effect of sea water. Thus any chemical warfare agent must be extremely potent, as it has to overcome dilution en route to its target.

Comprehensive research into the medicinal properties of marine organisms in 1960s examined a large number of shellfish products, particularly with the aim to isolate possible natural compounds for cancer treatment. Molluscs represent good candidates for anti-cancer natural products research considering their evolutionary and ecological significance. Currently, natural products isolated from molluscs and their structural analogues are particularly well represented in the anticancer compounds in clinical trials [4].

The species *P. viridis*, commonly known as the Indian green mussel, is a widely distributed edible mytilid bivalve seen all along both east and west coast of India. Even though many works have been undertaken with regard to pollution studies as a sentinel organism, *P. viridis* is been sparsely included in pharmacological studies. Some previous works conducted of this kind include, inhibition of osteoclasts formation [5], inhibition of replication of *Plasmodium faciparum* [6] and inhibition of HIV virus replication [7] etc. Some authors have also reported anti-microbial [8 & 9], radioprotective [10] and anti-angiogenic [11] and anti-inflammatory [12] properties of this mussel recently. However, no attempts were made to find out its cytotoxic properties so far. Therefore, the present work is first of its kind investigating the free radical scavenging and cytotoxic properties of the ethyl acetate extract of the species employing various assays.

MATERIALS AND METHODS

Chemicals

DPPH was purchased from Sigma chemical Co (USA), BHT (Butylated Hydroxy Toluene) and DMSO was obtained from Merck. All the other chemicals used were of analytical grade.

Collection of specimen

The mussel *Perna viridis* was collected from its natural bed at Anthakaranazhi, Alappuzha District (Kerala, India). They were brought to the lab in aerated plastic containers filled with sea water of ambient salinity. Mussels of both sexes were selected and washed in a jet of water and cleaned thoroughly to get rid of the attached algae and debris.

Extraction

The shells were separated in lab and the whole mussel tissue weighing 300 g was macerated with ethyl acetate (EtOAc) in a blender. The mixture was subjected to mechanical stirring overnight at room temperature and the suspension was centrifuged at 8000 rpm for 20 min. Resultant residue was treated with more solvent and the whole process was repeated two or more times. The supernatant solvent was concentrated using vacuum evaporator (35-55^{o}C) under reduced pressure and the resultant extract was weighed and kept in clean glass vials at -80 ^{o}C until use.

Preliminary Identification of the chemical components using general detection reagents

Identification of the chemical constituents (alkaloids, flavonoids, phenolics, saponins and sterols) present in the ethyl acetate extract

of *P. viridis* were carried out using various general detection reagents as described by Cannell [13].

Spray reagent detection of the components of extract on TLC

Further qualitative evaluation of ethyl acetate extract of *P. viridis* was done using thin layer chromatography (solvent system; Hexane: ethyl acetate (70:30)) and the R_f values were noted. The separated components were subjected to spray reagents specific to different secondary metabolites like alkaloids, phenolics, steroids and terpenes [13].

DPPH radical scavenging assay

The free radical scavenging activity of EtOAc extract of *P. viridis* was assessed using DPPH (1,1-diphenyl-2-picryl hydrazyl) radical[14]. Due to the poor solubility of EtOAc extract in aqueous medium it was dissolved in 1 % DMSO and later on made up to desired concentrations with distilled water. The extract in different concentrations were added to 0.1 ml of 1 M Tris–HCl (p^H 7.9), and then mixed with 0.6 ml of DPPH (100 µM) in methanol for 20 min at room temperature under protection from light. Absorbance of the mixture was read on a UV-VIS spectrophotometer (Hitachi, U-2001) at 517nm against the blank. Butylated Hydroxyl Toluene (BHT) was used as the standard antioxidant and the percentage inhibition of test and the standard were calculated as

$$\% \text{ Inhibition} = ((A_{Control} - A_{Test}) / A_{Control}) \times 100$$

where $A_{Control}$ is the absorbance of the control (without extract) and A_{Test} is the absorbance of the sample of extract and standard.

Brine shrimp lethality bioassay

The dried cysts of brine shrimps (Artemia sp.) were hatched in a shallow rectangular glass dish filled with filtered sea water. After hatching, the phototrophic nauplii were collected using a pipette from the lighted side. Ten nauplii each were transferred to vials containing different concentrations (30-225 µg/ml) of EtOAc extract dissolved in DMSO (1%) and the total volume was made up to 5 ml using sea water of 30 ppt. A control vial with 1 % DMSO was also maintained. A drop of dry yeast suspension was added as food to each vials and maintained under illumination. The experiments were done in triplicate. Number of survivors was counted after 12 h and 24 h and the percentage of death at each dose and control were determined [15]. Larvae were considered dead if no movement of the appendage was observed within 10 seconds.

Cytotoxic studies on DLA and EAC cells

In vitro cytotoxicity of the EtOAc extract was studied using the ascitic tumour cells such as Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC). Tumour cells were maintained in mice and aspirated under aseptic conditions. The cells were aseptically withdrawn and washed in PBS to remove traces of blood. One million cells in 0.5 ml PBS was incubated with varying concentrations of EtOAc extract (50-800 µg/ml) dissolved in 1% DMSO in a total of 1ml PBS at 37 ^oC for 3 hours. After incubation the cell death was evaluated using trypan blue exclusion method [16]. The stained and unstained cells were counted separately in a haemocytometer and the percentage cell death was calculated.

MTT assay on HCT 116 and MCF 7 cell lines

Cytotoxicity of the EtOAc extract of *P. viridis* was tested against two human cancer cell lines namely, HCT 116 (human colon adenocarcinoma) and MCF 7 (mammary adenocarcinoma) [17]. The cell lines were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FCS. The cells (5 X 10^3 cells (100 µl) per well) were allowed to attach for 24 h incubation at 37 ^oC in 5% CO_2 in 96-well plates. EtOAc extract (dissolved in 1% DMSO) at the rate of 50 and 100 µg/ml was added to each well and allowed to incubate for 24 h. Corresponding controls received 1% DMSO alone without the extract. On completion of incubation 100 µl (5 mg/ml) of MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added and incubated for 1 h. Subsequently the medium was removed; cell layer was washed with PBS, lysed in triplex solution and further incubated for 2 h. The suspension was centrifuged at 2500 rpm and the optical density of each sample well was read on 96 well plate reader (Bio Rad) at 570 nm. Six replicates were set up for each concentration and the percentage growth inhibition was calculated.

Statistical analysis

Statistical analysis was done using SPSS version 14. IC_{50} and LC_{50} values respectively for DPPH and cytotoxic assays were found out by Linear Regression Probit analysis.

RESULTS

Extraction and preliminary identification using general detection reagents

The ethyl acetate extract of *P. viridis* yielded a brown viscous mass with a percentage yield of 1.8%. Result of the preliminary identification of secondary metabolites is given in Table 1.

Spray reagent detection on TLC

The TLC plate of EtOAc extract developed with solvent system, hexane : EtOAc; 7:3, v/v displayed a coloured chromatogram with seven distinct visible bands E1 to E7 which were named from bottom to top. The R_f values of each bands are shown in Table 2. Spray reagent detection on these TLC plates revealed different organic constituents such as alkaloids, phenolics, terpenes and steroids (Table 3).

Table 1: Qualitative chemical evaluation of the EtOAc extract of *P. viridis* using general detection reagents

Test for the presence of chemical constituents	Ethyl acetate extract of *P. viridis*
Alkaloids	
Dragendorff's reagent	+ve
Poly phenols	
5 % $FeCl_3$	+ve
Sterols	
Liebermann-Buchard test	+ve
Salkowski reaction	+ve
Saponins	
Presence of persistent froth	-ve

Table 2: R_f values of ethyl acetate extract of *P. viridis* on TLC

Separated bands of EtOAc extract	R_f values
E1	0.17
E2	0.28
E3	0.50
E4	0.60
E5	0.71
E6	0.83
E7	0.93

Table 3: Compounds detected in EtOAc extract of *P. viridis* using different spray reagents on TLC

Compounds detected	Separated components of ethyl acetate extract
Alkaloids	E1 & E2
Phenolics	E4
Terpenes	E5
Steroids	E7

Table 4: LC_{50} values of EtOAc extract of *P. viridis* against different cytotoxic assays (lower and upper bound at 95% confidence interval are given in parentheses)

Assays	LC_{50} (µg/ml)	
Brine shrimp lethality	**12 h**	**24 h**
	176	92.4
	(163.2 - 2.7)	(74.5 - 110.5)
DLA	137.8	
	(88.6 - 192.9)	
EAC	311.5	
	(234.5 - 416.6)	

DPPH radical scavenging activity

DPPH radical scavenging property of *P. viridis* is shown in Figure 1. As depicted in the graph, the extract showed a dose dependent pattern in DPPH radical scavenging in the range of concentrations tested (0.15 – 6 mg/ml). The IC_{50} value of the extract was found to be 0.616 mg/ml whereas it was 0.343 mg/ml for the standard BHT. The higher concentration of EtOAc extract showed a percentage inhibition of 88.2 %.

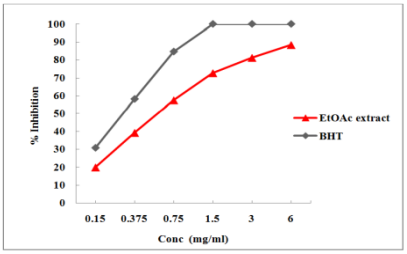

Figure 1: Effect of EtOAc extract of *P. viridis* and BHT on DPPH radical scavenging activity. Each value represents mean ± SD (n=3).

Effect on brine shrimp lethality

Incubation of the hatched artemia nauplii with EtOAc extract of *P. viridis* showed a dose dependent mortality rate (Figure 2) and the LC_{50} values were 176 and 92.4 µg/ml respectively for 12 and 24 h experiments (Table 4). Mortality was higher at 24 h incubation period than 12 h and 93% inhibition was observed for the higher dose, 225 µg/ml of EtOAc extract at 24 h incubation.

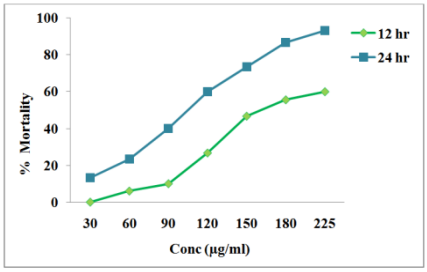

Figure 2: Effect of EtOAc extract of *P. viridis on* brine shrimp lethality. Each value represents mean ± SD (n=3).

Effect on DLA and EAC cells

The EtOAc extract showed a dose dependent inhibitory activity against DLA and EAC cells at a dose range of 50-800 µg/ml. The percentage inhibition corresponding to each dose level is shown in Figure 3. The higher concentration, 800 µg/ml produced an inhibition of 88.9 % and 99% respectively for EAC and DLA. Higher inhibition was observed against DLA than EAC; with an LC_{50} value of 137.8 and 311.5 µg/ml respectively for each cell (Table 3).

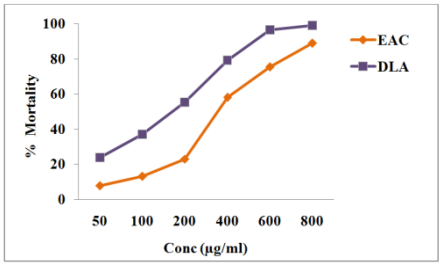

Figure 3: Effect of EtOAc extract of *P. viridis on* DLA and EAC cells. Each value represents mean ± SD (n=3).

Effect on HCT 116 and MCF 7 cell lines

EtOAc extract exhibited significant cytotoxicity against two human cancerous cell lines; MCF 7 and HCT 116 (Figure. 4). The extract was found to be more inhibitory towards MCF 7, with a percentage inhibition of 53.4 and 72.6 % respectively for 50 and 100 µg/ml tested. Upon treatment, HCT 116 cell line showed an inhibition of 63.7 % for 100 µg/ml of EtOAc extract.

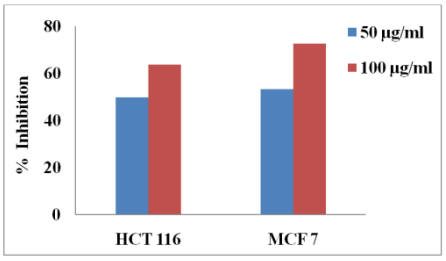

Figure 4: Cytotoxity of EtOAc extract of *P. viridis* on HCT 116 and MCF 7. Each value represents mean ± SD (n=6).

DISCUSSION

The chemical composition of EtOAc extract of *P. viridis* was found to be complex as judged by thin layer chromatographic separation and spray reagent detection. This revealed the presence of diverse groups of secondary metabolites like alkaloids, polyphenols, terpenes and sterols. Since phenolic compounds have been seldom reported from mlolluscs, their presence in *P. viridis* tissues was expected because these natural molecules are relatively easy to assimilate [18, 19]. Alkaloids have been isolated in reasonably large numbers from both classes (gastropods and bivalves) of molluscs, whereas aliphatic nitrogen containing compounds are relatively uncommon [20].

The results showed a concentration dependent activity of EtOAc extract of *P. viridis* extracts in scavenging DPPH radicals. The extract was able to reduce stable radical DPPH to the yellow coloured diphenylpicrylhydrazine, which was visually noticeable by the discolouration of test samples from purple to yellow. The decrease in absorbance caused by the antioxidants in the extracts might be due to the hydrogen donating ability resulted in the scavenging of the radical. Recent study on the methanolic extract of *P. viridis* showed significant radical scavenging property against DPPH radical [21].

Different cytotoxic assays proved, the potent cytotoxic nature of EtOAc extract of *P. viridis*. The extract exhibited significant level of mortality against brine shrimp lethality assay with respect to the time of exposure. Brine shrimp assay is considered as a useful tool for the preliminary assessment of toxicity and correlates reasonably well with cytotoxic and anti-tumour properties [22]. The assay indicates cytotoxicity as well as a wide range of pharmaceutical activities (anti-cancer, antiviral, insecticidal, pesticidal and AIDS etc) of the compound.

Considerable difference in brine shrimp mortalities were noted on treatment with EtOAc extract for 12 and 24 h experiments. In the case of 12 h exposure, effect of extract was comparatively less than that of 24 h. This can be due to the sensitivity of nauplii towards the test compounds in due course of time. As shown by Lewis [23] the nauplii exhibit their greatest sensitivity to test compounds during their second and third instar stage (24 h) in their life cycle. Which holds good considering the present results that instar II-III larvae (24 h) are significantly more sensitive which is further confirmed by the findings of the previous researchers [24, 25 & 26].

Cytotoxicity is one of the chemotherapeutic hallmarks of anti-tumour activity [27] and the ability of cells to survive a toxic insult has been the basis of most cytotoxicity assays. The *in vitro* cytotoxic activity of EtOAc extract of *P. viridis* against the murine tumour cells showed that it is more inhibitory towards DLA than EAC. These observed differences in cell line specificity, indicate that bioactive compounds of the extracts target alternative pathways depending on cell phenotype. Similar observations were also reported by Latha and Panikkar [28] and Radha *et al* [29]. Reduced ability of the tumour cells to survive after a cytotoxic insult can be observed directly by microscopic examination using a haemocytometer.

MTT assay is a well established *in vitro* model used to test cytotoxicity of compounds against cancer cell lines. It was used as one of the conventional method for screening of compounds with potential anti-tumour properties [30] and to test the effectiveness of carcinostatic agents [31]. The assay is based on the principle that viable cells convert the soluble yellow tetrazolium salt, the MTT compound (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl- tetrazolium bromide) to an insoluble purple coloured formazan precipitate by the mitochondrial enzyme succinate dehydrogenase whereas the dead cells cannot. Therefore the untreated wells show higher absorbance than the treated wells at 570 nm due to the formation of formazan, which can be correlated with the number of viable cells in the culture. The incubation of cell lines for 24 h with EtOAc extract resulted in cell death indicated by the decrease in absorbance at 570 nm.

Preliminary chemical evaluation of the EtOAc extract showed the presence of secondary metabolites like alkaloids, poly phenols and sterols. Poly phenols were reported to have an important role in stabilizing lipid peroxidation and are associated with a wide range of biological activities including antioxidant properties [32]. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity [33] with possible interaction with cell wall and DNA. Therefore from the above results it can be assumed that the presence of these components may have contributed to these activities of the extract.

CONCLUSION

Current study on the cytotoxic activity of EtOAc extract of *P. viridis* showed potent activity against brine shrimp assay, DLA and EAC cell lines with appreciable antioxidant property too. It also proved cytotoxic towards human cell lines (HCT 116 & MCF 7) which makes it a prospective candidate for future research. The extract tested positive to the presence of secondary metabolites like alkaloids, polyphenols and terpenes, hence it can be attributed that the aforesaid activity can be due to the presence these compounds.

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