Journal of Aquatic Biology & Fisheries | 2022 | 10(1) | pp. 67-70
ISSN 2321–340X
OPEN ACCESS
http://www.jabf.in/
University of Kerala, INDIA

In vivo anti-tumour activity of the green mussel *Perna viridis* extract on ascites and solid tumour models in mice

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ABSTRACT

Mussels contribute substantially to marine natural product discovery and have produced many active compounds in clinical trials in the past few decades. The present study aims to discover the potential anti-tumour activity of ethyl acetate extract of green mussel, *Perna viridis*, using ascites and solid tumour models in mice. The mussel extract was found to be effective in reducing the ascitic tumour in mice and increased the life span of tumour-bearing mice by 52.3, 64.5 & 77.6 %, respectively, for 62.5, 125 & 250 mg/kg doses. *P. viridis* extract also reduced the solid tumour formation in mice induced by injecting DLA cells ($1x10^6$ /0.5ml subcutaneously. A dose-dependent reduction in tumour volume was observed in treated mice compared to the control group. From the results, it can be concluded that the ethyl acetate extract from *P. viridis* could be a potential candidate among anti-cancer marine natural products in future.

ARTICLE HISTORY

Received on: 14-08-2022
Revised on: 04-09-2022
Accepted on: 22-12-2022

KEYWORDS

Perna viridis, Extract, Antitumor activity, Ascites, Solid tumour, DLA

1. Introduction

Phylum Mollusca displays great morphological, ecological, and chemical variability and chemical investigation on them has led to the isolation of a wide variety of bioactive metabolites, helping them to survive in different environments. Natural products have always played a major role in anti-cancer medicine, and molluscan-derived natural products and their structural analogues are particularly well represented in the anti-cancer compounds in clinical trials (Simon *et al.*, 2006). Moreover, in South Africa (Herbert *et al.*, 2003), India (Prabhakar & Roy, 2009) and China (Hu, 1980), molluscs are featured in a number of traditional medicines. Several molluscan-derived remedies are also recorded on the homoeopathic Materia Medica Rothman, 1984).

Comprehensive research in the 1960s on the bioactive properties of marine organisms, particularly with the aim to isolate the active agents observed in a large number of shellfish products, for cancer treatment. Considering their evolutionary and ecological significance, molluscs are found to be good candidates for anti-cancer natural products research. Natural products isolated from molluscs and their structural analogues are particularly well represented in the anti-cancer compounds in clinical trials (Simmons *et al.,* 2005). Marine mussels are an inexpensive source of protein, polyunsaturated fatty acids (PUFAs), and essential minerals, trace metals, and certain vitamins with valuable pharmaceutical and biomedical potential.

Perna viridis, commonly known as Indian green mussel, is a widely distributed species seen along India's east and west coasts. This species has been widely studied for its biological and cultural aspects (Sreenivasan *et al.,* 1989; Rivonker *et al.,* 1993; Rajagopal *et al.,* 1998). Many researchers also reported its medicinal properties such as anti-microbial (Annamalai *et al.,* 2007; Chandran *et al.,* 2009), Anti-bacterial (Madhu *et al*, 2014), anti-angiogenic (Mirshahi *et al.,* 2009), anti- inflammatory (Sreejamole *et al*., 2011; Joshi *et al.,* 2020), cytotoxic (Sreejamole & Radhakrishnan, 2013), antioxidant (Sreejamole & Radhakrishnan, 2016; Chakraborty *et al*., 2019) and anti-cancer (Praveena & Fathima, 2017) properties.

The present study investigates *in vivo* anti-tumour activities of *Perna viridis* extract using both ascites and solid tumour models in mice.

2. Materials and Methods

2.1. Collection and extraction

The mussel *Perna viridis* was collected from Anthakaranazhi, Alappuzha District (Kerala, India) and brought to the lab in aerated plastic containers filled with sea water of ambient salinity. Mussels were washed in water and cleaned thoroughly to get rid of attached algae and debris. Shells were separated in the 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. The resultant residue was treated with more solvent and the whole process was repeated two or more times. The supernatant solvent was concentrated using a 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.

2.2. Animals

Swiss albino mice of age group 8-10 weeks weighing 22-26 g, maintained under conventional laboratory conditions, were used in all experiments. Mice were fed with a standard mouse diet (Lipton, India) and water *ad libitum.* Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) were maintained as ascites by weekly serial transplantation through intraperitoneal(i.p.) injection of 1x 10^6 cells/ mouse. The tumour cells were aspirated

aseptically from the tumour-bearing mice and washed thrice in PBS (phosphate-buffered saline) before transplantation. All animal experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of the Committee (Registered Number 149/1999/CPCSEA India/28/2/2004).

2.3. Ascites tumour experiment on rats

In vivo anti-tumour studies of the EtOAc extract of *P. viridis* were carried out with DLA ascitic and solid tumour models in mice. Mice (20-30 g) were divided into four groups of ten animals each. All four groups were transplanted i.p with $1X10^6$ DLA cells. After 24 hours, group I, serving as control, was given 0.5 ml 10% DMSO, and groups II, III and IV received orally 0.5 ml of EtOAc extract at Conc. 62.5, 125 and 250 mg/kg respectively. Administration of extract was continued once daily until the death of all the control animals, and the mortality was noted every day up to 45 days. The growth inhibitory effect on DLA tumour cell implanted mice was evaluated by the determination of animal survival expressed as mean survival time (MST) in days. The percentage increase in life span (% ILS) was calculated as described by Nair and Panikkar (1990) using the formula

$$\% \text{ ILS} = \frac{T - C}{C} \times 100$$

Where 'T' is the mean survival time in days of the treated group and 'C' is that of control. The body weight of animals of each group was noted before and after tumour induction up to 28 days.

2.4. Solid tumour experiment on rats

For solid tumour induction, mice were divided into five groups of six animals each. DLA tumour cells ($1x\ 10^6$/0.5 ml) were subcutaneously injected into the right hind leg of the mice to form the solid tumour (Nair and Panikkar, 1990). Control animals received 0.5 ml of 10% DMSO and all the other groups received the EtOAc extract at three different concentrations (62.5, 125 and 250 mg/kg) 24 h after DLA implantation upto 10 days. The radii of the developing tumour were measured using vernier callipers at 5-day intervals up to 30 days. The tumour volume was determined using the formula,

$$V = 4/3\ \pi\ r_1^2 r_2.$$

where r_1 and r_2 are the radii of the tumour along two directions.

3. Statistical analysis

Statistical analysis was done using SPSS version 14. The mean survival time of the ascites tumour-induced mice and their comparison were made by Kaplan- Meier analysis. For anti-tumour assays, General Linear Model Univariate analysis followed by Dunnett's t-test as post-Hoc were carried out. *p value* <0.05 was considered significant.

4. Results

4.1. Effect on ascites tumour reduction

Palpable ascitic tumours appeared in the peritoneal cavity of the mice, within 7-12 days of i.p. injection of DLA tumour cells. Anti-tumour activity of the EtOAc extract of *P. viridis* was measured by the survival rate and % ILS after the transplantation of DLA tumour cells. In the case of control mice, the tumour grew progressively and the survival time was significantly reduced (21.4±1.12 days) compared to the EtOAc extract treated mice (Table 1). Administration of EtOAc extract significantly reduced murine ascitic tumour growth and increased the life span of tumour bearing animals by 52.3%, 64.5% and 77.6%, respectively, for 62.5, 125 and 250 mg/kg doses (Fig 1). The mean survival time of treated groups was significantly different from each ($p<0.001$) by Kaplan Meier analysis.

Table 1. Effect of EtOAc extract of *P. viridis* on the life span of DLA tumour bearing mice

Treatments	Mean survival time (MST) in days±S.E
Control	21.4±1.12
EtOAc extract (62.5 mg/kg)	32.6±1.74***
EtOAc extract (125 mg/kg)	35.2±2.44***
EtOAc extract (250 mg/kg)	38±2.08***

The potent anti-cancer nature of the extract was also evident from the significant reduction in body weight gain compared to control in treated groups. Treating with EtOAc extract (125 and 250 mg/kg) significantly ($p<0.001$) reduced the body weight at 14 and 28 days compared to the control group treated with 10 % DMSO alone (Fig. 2). Control group showed higher mean weight increase, relative to the initial weight than the treated groups. An overall significant ($p<0.001$) difference in body weight was observed between the groups for treatment and days of treatment.

4.2. Effect on solid tumour reduction

Administration with EtOAc extract of *P. viridis* significantly inhibited the growth of subcutaneously transplanted DLA solid tumours and delayed the onset of tumour formation. The tumour volume of the control group showed a progressive increase and reached 4.05±0.36 cc on day 30, whereas the tumour volume of the treated groups was 3.7, 3.45 and 2.88 cc, respectively (Fig 3). Same as in the case of ascitic tumours , here too, a dose dependent effect on tumour reduction was observed starting from day 5 to 30. A significant ($p<0.001$) difference in solid tumour volume was noted by univariate analysis for treatment and days. Multiple comparisons by Dunnett's t-test revealed that

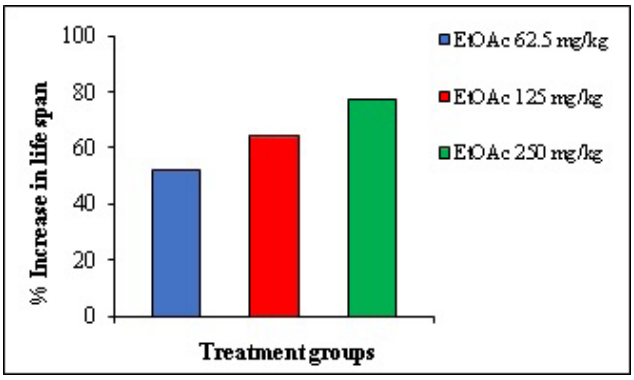

Fig. 1. Effect of EtOAc extract of *P. viridis* on the percentage life span of DLA ascitic tumour induced mice

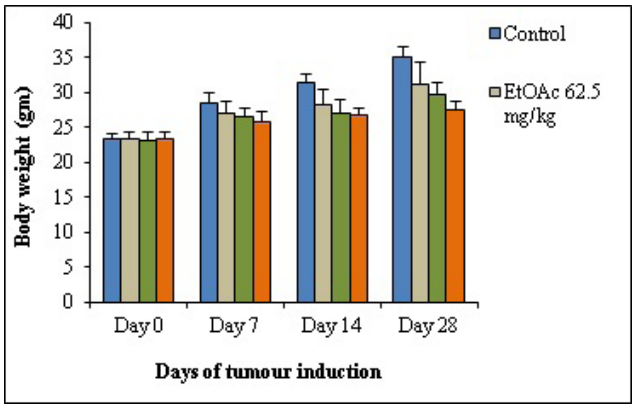

Fig. 2. Effect of EtOAc extract of *P. viridis* on the body weight of DLA ascitic tumour induced mice

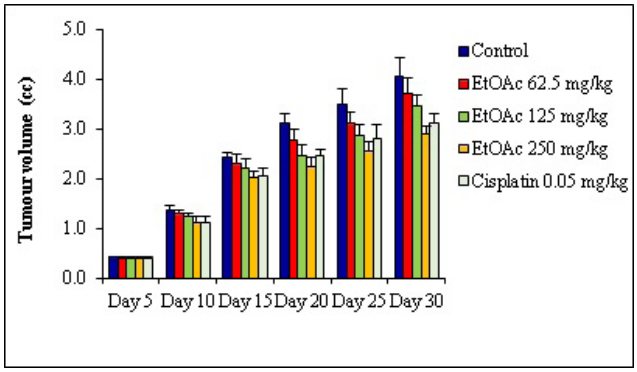

Fig. 3. Effect of the EtOAc extract of *Perna viridis* on the growth of DLA solid tumour in mice

the EtOAc extract treated groups (125 and 250 mg/kg) significantly reduced ($p \leq 0.001$) the tumour volume from the 15th day onwards compared to the control.

5. Discussion

The reliable criteria for judging the value of an anti-cancer drug are the prolongation of life span and inhibition of gain in average body weight (Oberling and Guerin, 1954; Clarkson and Burchnel, 1965). Treatment with *P. viridis* extract inhibited the gain in body weight and reduction in ascitic fluid accumulation in the peritoneal cavity in comparison with the control animals. The suppression of tumour growth and the reduction in body weight were also reported for liposome incorporated aqueous extracts of *Anodonta woodiana* against human QGY hepatic carcinoma and MKN-45 gastric tumours inoculated mice during 30 days (Liu *et al.,* 2008).

A significant increase in the life span of the tumour induced animals on treatment with EtOAc extract implies the potent anti-cancer properties of the extract. These results could indicate either a direct cytotoxic effect of the extract, which was earlier reported in one of our studies on different cell lines (Sreejamole & Radhakrishnan, 2013). It can also be suggested that the reduction in nutritional fluid volume arrested the tumour growth, thereby increasing the life span of DLA bearing mice. The EtOAc extract was also found to inhibit solid tumour volume in a dose dependent manner. This reduction in tumour volume indicated that EtOAc extract plays a direct role in killing the tumour cells and thereby enhances the curative effect on the tumour induced animals.

A study on the extracts prepared from Indian green mussel (*Perna viridis* L.) has shown to inhibit the formation of endothelial cell capillary tubes in a concentration-dependent manner *in vitro* (Mirshahi *et al.,* 2009). Substances or molecules, having anti-angiogenic properties are useful in cancer therapy, and these substances can be used to inhibit the growth of tumour mass by preventing neo-vascularization of an early developing tumour. This result can be correlated with the present findings in inhibiting solid tumour formation by *P. viridis* extracts.

It has been found that related species, *P. canaliculus* commonly referred to as New Zealand green lipped mussel shown to inhibit prostate cancer, estrogen dependent or non-estrogen dependent breast cancer, melanaoma and bladder cancer (Kendall and Lawson, 2004). The molecular basis of the activity was supposed to be the inhibition of some stages of cell cycling, probably the S phase. The component responsible for the activity is suggested to be a lipid or carbohydrate fraction. In addition, a number of studies have been reported showing cytotoxic and anti-tumour properties in species like *Patinopecten yessoensis, Chlamys farreri* and *Cristaria plicata* (Takuma *et al.,* 1987; Gu *et al.,* 1998; Tong *et al.,* 2002).

6. Conclusion

The overall results of the present study show a significant cytotoxic and tumour inhibitory activity of EtOAc extract of *P. viridis,* augmenting the life span of tumour-induced animals. The aforesaid activity of the extract can be attributed to the direct cytotoxic effect on the tumour cells. These findings highlight the biomedical potential of *P. viridis* extracts in the development of a natural therapy for the treatment of neoplastic tumours and lymphomas. However, the exact mechanism through which the apoptosis is persuaded, and the nature of the active component(s) are yet to find out.

Acknowledgement

We are grateful to Amala Cancer Research Centre, Trichur, for providing all the facilities for conducting this work.

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