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Evaluation, partial characterization and purification of acetylcholine esterase enzyme and antiangiogenic activity from marine sponges

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PEER REVIEW

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Comments

The paper deals with an important argument, cause of the multiple applications and functions of the studied enzyme. The experiments are carefully described and the figures are convincing.
Details on Page 853

ABSTRACT

Objective: To test three marine sponges *Halichondria glabrata* Keller, 1891; *Spirastrella pachyspira (S. pachyspira)* Levi, 1958 and *Cliona lobata* Hancock, 1849 for the presence of the acetylcholinesterase (AChE) in both young and developed samples from western coastal area of India. *S. pachyspira* methanolic extract was selected for anti/pro angiogenic activity.
Methods: They were evaluated for AChE activity using Ellman's assay based on production of yellow colored 5-thio-2-nitrobenzoate. Purification of the enzyme was planned using ammonium sulphate precipitation and characterization by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Chorioallantoic membrane (ChAM) assay model was used for angiogenic/antiangiogenic testing.
Results: All the three sponges showed good specific enzyme activity and *S. pachyspira* contained maximum specific enzyme activity. Sixty percent of ammonium sulphate precipitation of crude protein sample gave single band at 66 kDa corresponding to the true AChE. ChAM assay was performed at 62.5, 125.0 and 250.0 μg/mL. Dosage beyond 250 μg/mL extract showed toxic response with anti angiogenic activity at all the concentrations.
Conclusions: AChE activity was detected in all samples. Extract showed good anti-angiogenic response at 62.5 μg/mL. Extract was highly toxic affecting microvasculature of ChAM as well as normal growth and development of the embryo at 500 μg/mL. With further characterization of bioactive compounds from the extract of *S. pachyspira*, the compounds can be developed for anti tumor activity.

KEYWORDS

Halichondria glabrata Keller, *Spirastrella pachyspira* Levi, *Cliona lobata* Hancock, Acetylcholinesterase, Chorioallantoic membrane, Antiangiogenic activity, Sodium dodecyl sulfate polyacrylamide gel electrophoresis

1. Introduction

Marine sponges (Porifera) are one of the oldest metazoan groups, having a remarkable importance as a living fossil. They are a rich source of chemically novel products with a broad spectrum of bioactivity and many compounds derived from these organisms have generated interest for their cytotoxicity. There are about 8 000 described species of sponges and perhaps twice as many undescribed species[1]. Countless applications for these species have been identified and explored for bioactivity, including, the inhibition of tumor cell growth, anti-viral activity against hepatitis B and the treatment of Alzheimer's disease[2]. In the sympathetic and parasympathetic nervous systems and at all neuromuscular junctions, acetylcholine is used to signal muscle movement. If this neurotransmitter is not broken down after serving its function, the muscle involved would not be able to relax, and this could create spasms, paralysis, and other problems. Just as the transmission of nerve impulse is important, its termination is equally

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important because the continuous transmission of nerve impulse would lead to heart failure and finally to the death of the individual. The enzyme acetylcholinesterase (AChE) has this important function which helps to terminate the flow of acetylcholin (ACh) by hydrolyzing it to give choline and acetate moieties. In the absence of AChE, the signal cannot be turned off and it continues unabated till the heart fails[3]. Because of the pivotal role played by AChE in the transmission of nerve impulse, an understanding into the mechanism of action of AChE is also important to develop AChE inhibitors. While the level of AChE and its molecular species are altered in the Alzheimer's disease brain, AChE activity in the cerebrospinal fluid has also been measured in assessing the pathophysiology of Alzheimer's disease. The emerging consensus is that total cerebrospinal fluid -AChE levels decrease modestly as dementia progresses. Measurement of AChE levels in Alzheimer's disease might have some value in monitoring disease progression and is still of interest due to increasing evidence linking β-amyloid processing and AChE activity[4]. Assay of AChE activity plays an important role in diagnostic, detection of pesticides and nerve agents, *in vitro* characterization of toxins and drugs including potential treatments for Alzheimer's disease. Apart from these regular roles, AChE is also said to play roles as an adhesion protein in the synaptic maintenance and development[5] and have evidence in neurite growth[6]. An inhibition of this enzyme has been used to detect and measure the biological effects of organophosphorus and carbamates pesticides in the marine environment[7]. It can also be inhibited by the presence of heavy metals and surfactants[8]. Many marine-derived natural products and their analogues have been reported to show antiangiogenic activities. As the growth of the tumor could be stopped by blocking the development of new blood vessels with the help of antiangiogenic drugs[9], the natural resources especially marine sponges are worth exploring for antiangiogenic substances which holds great promise. At least 43 marine-derived natural products and their derivatives have been reported to display antiangiogenic activities, mediated by distinct or unknown molecular mechanisms. The diverse chemical structures confer distinct activities and mechanisms of action to main classes: protein kinase modulators, cytoskeleton disturbing agents, histone deacetylases inhibitors, methionine amino peptidase inhibitors and others based on their mechanisms/primary targets[10]. Recognition that control of angiogenesis could have therapeutic value has stimulated great interest during the past 40 years. Stimulation of angiogenesis can be therapeutic in ischemic heart disease, peripheral arterial disease, and wound healing. Decreasing or inhibiting angiogenesis can be therapeutic in cancer, ophthalmic conditions, rheumatoid arthritis and other diseases. Analogues inspired by marine secondary metabolites (using dibromotyrosine as precursor) were tested by Sallam *et al*.[11] using biological models to determine the antiangiogenic effects, including the chorioallantoic membrane (ChAM) assay, wound-healing assay and basement membrane extract cell invasion. These analogues showed antiangiogenic activity and inhibition of prostate cancer cell proliferation and migration[11]. A novel matrix metalloproteinase inhibitor, ageladine A with antiangiogenic activity was isolated from a marine sponge *Agelas nakamurai* later identified as 4-(4,5-dibromo-1H-pyrrol-2-yl)]-1H-imidazo[4,5-c] pyridin-2-amine[12]. Smenospongine, from a marine sponge *Dactylospongia elegans*, exhibited antitumor activity on solid tumors via two mechanisms, an antiangiogenic effect on endothelial cells and direct inhibition of growth of tumor cells[13]. The main objective of our study is to investigate the variation of AChE enzyme activity in three sponges species from different family collected from two pre-selected sites, along the Mumbai coast, considered undisturbed in terms of pollution, although some anthropogenic activities can occur.

2. Materials and methods

2.1. Sampling and identification

The sponges were collected from the coastal region of Mumbai, Khardanda (19°4′20″ N 72°49′52″ E) and Bandra fort, India by hand picking during the low tide. Samples were collected initially in the month of December 2012 when the sponges were in their initial stage and again, in the month of March 2013 during their developing stage. Both the samples were stored in freezer at -80 °C. The samples were sent to Zoological Survey of India, Chennai for identification.

2.2. Extraction

The marine sponges were homogenized in phosphate buffer (pH 7.0, 0.1 mol/L, containing 1 mmol/L ethylene diamine tetraacetic acid, 1% Triton X-100, 1 mol/L NaCl) and were then centrifuged at 10000 r/min for 30 min at 4 °C.

2.3. Ammonium sulphate precipitation of protein

Solid ammonium sulfates were added to 30%, 45% and 60% saturation on ice with gentle stirring. After solids completely dissolved, the suspension was centrifuged. Pellets were collected and dissolved, and dialysis was carried out.

2.4. Protein estimation

Total protein content of the sponge was analyzed by Folin-Lowry method[14].

2.4.1. AChE activity determination

The extraction of AChE was done by phosphate buffer with a suitable surfactant Triton which aided for good amount of enzyme. Further partial protein purification was done by ammonium sulphate precipitation, and 60% gave a single band of enzyme at 66 kDa in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). However it is reported to separate into 3 or 4 bands depending on the nature of the AChE in native polyacrylamide gel electrophoresis (PAGE)[15]. AChE activity was evaluated by Ellmans method which accounts for conversion of 5-5″-Dithio-bis (2-nitrobenzoic acid) to thionitrobenzoate and

monitors the reaction with AChE. This method was used since it is extremely sensitive and small quantities of sample can be used[16]. Repeatability studies and intraday variability for enzyme activity was done for all the three samples using 5-5''-Dithio-bis (2-nitrobenzoic acid) to thionitrobenzoate conversions.

2.4.2. Denaturing polyacrylamide gel electrophoresis

The molecular weight of protein may be estimated if they are subjected to electrophoresis in the presence of a detergent sodium dodecyl sulfate and a reducing agent mercaptoethanol. For denaturing polyacrylamide gel electrophoresis (SDS-PAGE), 12% resolving gel was overlaid with 4% stacking gel. Samples were run with constant voltage of 110 V. Molecular weight markers for SDS-PAGE were used for comparison[17].

2.5. Angiogenic/antiangiogenic activity on ChAM of chick embryo

Spirastrella pachyspira (S. pachyspira) was selected for this study because of strong enzyme activity. *S. pachyspira* sponge was soaked in methanol (1:10 ratio) for 5 h; the solvent was removed after squeezing the sponge. The filtration was done through Whatman Filter paper 1. The solvent was evaporated at low pressure using a Buchi Rotavapor at 40 °C and the concentrated extract was stored at 4 °C for further use. *S. pachyspira* extract was tested at 62.5, 125.0, 250.0 and 500.0 μg/mL. Fertilized crossbred eggs were obtained from Central Poultry Development Organisation, Goregoan, Mumbai. The ChAM assay was performed as described earlier[18]. They were incubated in humidified incubator for 5 d at 38 °C. On Day 5, eggs were candled to locate ChAM. The area was marked and window of 1 mm×1 mm was made. The marked window was cut in order to see the embryo and the surrounding blood vessels on ChAM. Extract of the desired concentration (volume 10 μL) was dissolved in (2.5%) agar. This solution was air dried on a Teflon-coated tray, and dried agar disks of 4 mm were prepared. Discs with sponge extract were placed on outer portion of ChAM which was visible through the window. Windows were sealed with parafilm and were kept in the incubator for 48 h. Angiogenic or antiangiogenic response was assessed by measuring densely vascular/a vascular zone around the disc respectively. A vascular zone larger than 4 mm in diameter was considered as positive. Results are presented as the percentage of embryos that showed antiangiogenesis in response to the treatment with sponge extract. A positive control (10 μg/mL) was kept using a mixture of 60 μg of hydrocortisone (Hi-media Laboratory, Cat. No. RM556) and 50 μg of heparin (Hi-media Laboratory, Cat. No. RM639). An agar disk with saline was used as negative control, which did not show activity. Fifteen eggs were used for each dose, and the experiment was performed in triplicate to ensure reproducibility.

3. Results

3.1. Identification of marine sponges

The samples were identified as *Halichondria glabrata (H. glabrata)* Keller, 1891; *S. pachyspira* Levi, 1958 and *Cliona lobata* (*C. lobata*) Hancock, 1849 by Zoological Survey of India, Chennai with registration Nos. S-167a, S-235 and S-236 respectively.

3.2. Protein estimation

The protein estimation was done by Folin-Lowry method. The different salt fractions were checked for protein concentrations as shown in Table 1. As the salt concentration increased, the protein in the precipitate increased. Crude protein extract of *C. lobata* showed highest protein concentration followed by *S. pachyspira* and *H. glabrata*. After precipitation almost all the samples had similar concentrations.

Table 1

Concentration of the protein from the sponge samples.

Sample	Protein (mg/mL)			
	Crude	Fraction A with 30% salt	Fraction B with 45% salt	Fraction A with 60% salt
C. lobata	48.7	20.6	21.9	24.8
H. glabrata	35.9	26.9	25.9	26.3
S. pachyspira	38.5	12.2	26.2	27.4

3.3. AChE characterization using denaturing polyacrylamide gel electrophoresis

One dimension SDS-PAGE was carried out on vertical slab gel system. The protein profiling was done for the crude and the purified samples. The results are shown in Figure 1. When SDS-PAGE was carried out, it showed a single band due to the conversion of the AChE into a monomer due to denaturing by sodium dodecyl sulfonate. If this extract is run over in a non-denaturing condition it gives 3 or 4 bands depending on the nature of the AChE[18]. The crude extract showed the presence of 4 bands inclusive of one for 66 kDa characteristic for AChE. The 66 kDa band is also specific for the M1 ACh receptor protein, as well as for serum albumin, and possibly other proteins, a Western blot using an anti-AChE antibody would solve the problem. With the partial purification of the sample with salt fractionation at 30% and 60% showed a clear band at 66 kDa. The 30% precipitate showed presence of other proteins while 60% precipitate showed a clear band at the 66 kDa. AChE is reported to have molecular weight of 260 kDa.

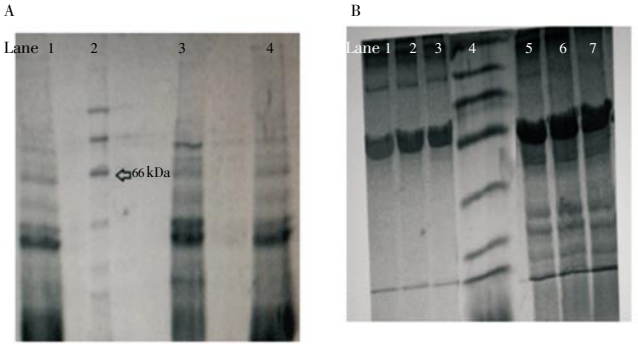

Figure 1. SDS-PAGE for crude and partially purified protein.

A: SDS PAGE for crude protein. Lane 1: *S. pachyspira*; Lane 2: Protein ladder; Lane 3: *C. lobata*; Lane 4: *H. glabrata*. B: SDS PAGE for partially purified protein. Lane 1: *S. pachyspira*; Lane 2: *C. lobata*; Lane 3: *H. glabrata*; Lane 4: Protein ladder; Lane 5: *S. pachyspira*; Lane 6: *C. lobata*; Lane 7: *H. glabrata*.

3.4. AChE enzyme activity determination

The Ellman's method for AChE activity determination is based on the rate of production of thiocholine, because

of the hydrolysis of acetylthiocholine. This reaction was monitored for the production of thiocholine with the DTNB ion, producing the yellow anion 5-thionitrobenzoate which was monitored at 412 nm[16]. Comparisons were done and the plot in Figure 2 clearly shows significant increase in the specific enzyme activity of all the three sponge samples in grown stage as compared to young. In repeatability and intraday variability studies for enzyme activity, the results of the young samples where variable, whereas grown sample enzyme activity was stable with no drastic fall in enzyme activity as represented in Figures 3 and 4. Enzyme activity was also checked for the partially purified enzyme. A comparison was done of the crude versus the partially purified sample. After purification, AChE activity was checked in both the precipitate and the supernatant. Results indicated enzyme activity only in the precipitate shown in Table 2. The enzyme activity did not vary much and activity was retained in the 60% salt precipitates.

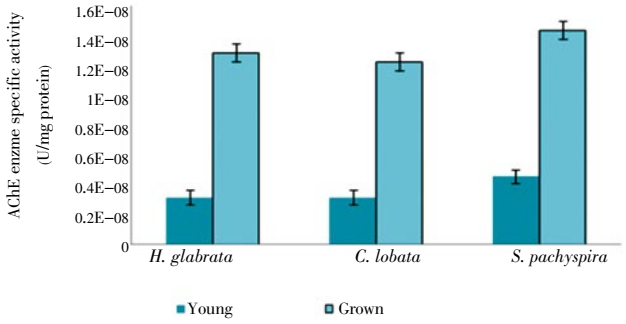

Figure 2. Comparative AChE specific enzyme activities between young and grown sponges.

Table 2

Enzyme activity of crude and partially purified samples.

Sample	AChE enzyme specific activity		
	H. glabrata	*C. lobata*	*S. pachyspira*
Crude	1.2852E-06	1.226E-06	1.604E-06
60% protein ppt	1.0453E-06	1.164E-06	1.427E-06

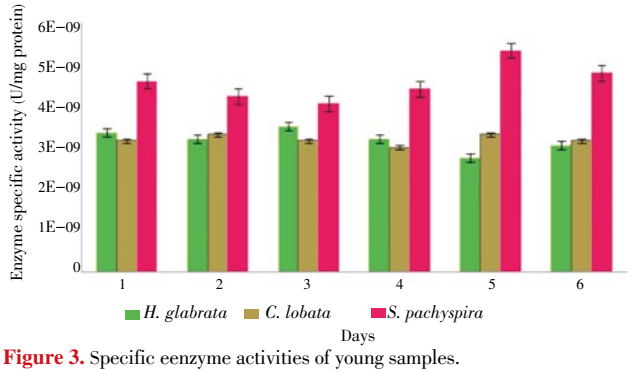

Figure 3. Specific eenzyme activities of young samples.

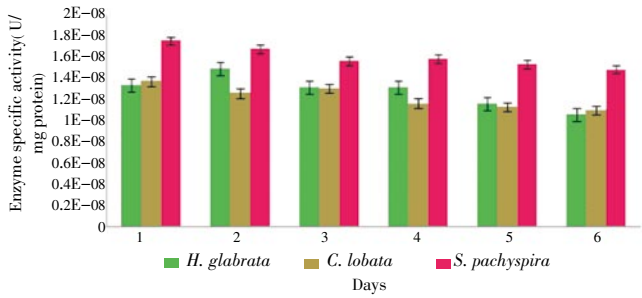

Figure 4. Specific enzyme activities of grown samples.

3.5. Angiogenic/antiangiogenic activity of extract on ChAM of chick embryo

All concentrations of extract were showing antiangiogenic response. Antiangiogenic response given by the *S. pachyspira* extract was dose-dependent (Table 3). The extract showed toxic response from 250.0 μg/mL onwards. At 250.0 μg/mL, development of embryo was affected and was underdeveloped. At 500.0 μg/mL, all the embryos were found to be dead due to toxicity of the extract. Figure 5 shows the images of marine sponge extract treated ChAM and the negative control.

Table 3

Effect of different concentrations of the *S. pachyspira* extract on ChAM

Conc. (μg/mL)	Activity	Activity/15 eggs	% Antiangiogenic response
Negative control	-	NA	-
Positive control	Antiangiogenic	Toxic	100
62.5	+	3	20
125.0	++	9	60
250.0	+++	9	60
500.0	Dead	Toxic	100

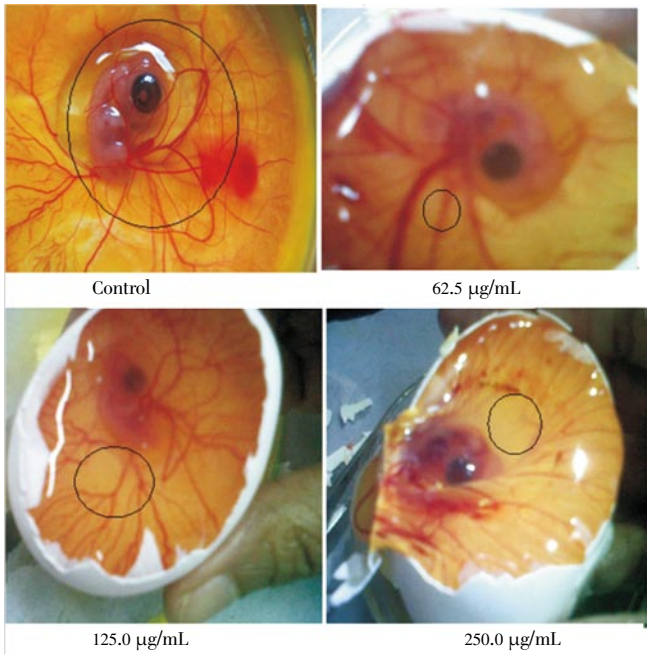

Figure 5. Micro-vasculature of negative control and treated ChAM by *S. pachyspira* extract (circled area indicates the position of agar discs on ChAM).

In control ChAM, the blood vessels were distributed in tree branches like patterns in which primary blood vessels gives off secondary blood vessels. At lower doses of 62.5, 125.0 and 250.0 μg/mL tertiary vasculature was present, but with the increase in dose the vasculature became torturous. Extract showed good antiangiogenic response at 62.5 μg/mL. Extract was highly toxic affecting microvasculature of ChAM as well as normal growth and development of the embryo at 500.0 μg/mL, which was probably due to extensive hemorrhage. Dose of 500.0 μg/mL was toxic and all the eggs died.

4. Discussion

Although the phylum Porifera are multicellular organism, they are generally considered to be primitive and

evolutionally. They lack nervous system but histochemical detection of AChE activity has provided indirect evidence of ACh expression in them a conclusion that is directly confirmed by the present study in three marine sponges belonging to Demospongiae group but different group and family[19]. Three sponges identified as *H. glabrata* Keller, 1891; *S. pachyspira* Levi, 1958 and *C. lobata* Hancock, 1849 are a new addition to the distribution of these species in the coastal areas of Mumbai, India. These sponges have not been studied for their AChE and antiangiogenic activities to the best of our knowledge. Although the function of ACh in sponges is not known, it might act as a local mediator, modulating the responses of independent effectors or spontaneously active cells so that nutrients are collected effectively. The capacity to synthesize ACh varies among the sponges and is unrelated to the ACh content. The possibility that ACh content in sponge is dependent on the cohabitants such as bacteria cannot be ruled out[20]. AChE is an enzyme essential to correct transmission of nerve impulses, and inhibition of this enzyme has been used to detect and measure the biological effects of organophosphorus and carbamates pesticides in the marine environment[7]. In an earlier work large polymeric 3-alkylpyridinium salts from the marine sponge-*Reniera Sarai* were isolated and acted as acetylcholinesterase inhibitors and showed an unusual inhibition pattern[21]. It was tentatively described as quick initial reversible binding, followed by slow binding or irreversible inhibition of the enzyme. Another recent study reports thin-layer chromatography and microplate assays revealing potent AChE inhibitory activities of two ethyl acetate extracts from the sponges *Pericharax heteroraphis* and *Amphimedon navalis*[22]. AChE inhibitors from marine sponges have been rarely studied, and this study demonstrates the potential of marine sponges present with AChE enzymes and probable inhibitors as a source of pharmaceutical leads against neurodegenerative diseases and also for cancer therapy, as it was demonstrated for *Haliclona sarai* and *Reniera sarai*[23,24]. To claim similar kind of inhibitors which might be responsible for antiangiogenic activity in *S. pachyspira*, further studies are required to evaluate and check AChE inhibitory activity. The antiangiogenic effect of marine organisms, make them a potential candidate in the development of new antiangiogenic drug in cancer therapy. In particular, marine sponge-derived antiangiogenic protein kinase modulators based on the critical roles of either tyrosine kinase or serine-threonine kinase in tumor angiogenesis and the tremendous marine resources are yet to be developed. In our study, the antiangiogenic activity of the crude extract of the sponge *S. pachyspira* was studied by performing ChAM assay. The extracts were highly toxic to the eggs at concentrations above 62.5 μg/mL. These concentrations of extract caused hemorrhage by reducing the blood supply. In some cases half of the embryo had reduced blood supply, whereas the other half was normal. The extract obtained from *S. pachyspira* was a potent angiogenesis inhibitor: it showed 20% activity at 62.5 μg/mL and 60% activity at 125.0 and 250.0 μg/mL. The mixture of hydrocortison and heparin, at (10 μg/mL) which was used as a positive control, and 500 μg/mL crude extract showed 100% antiangiogenic activity. The present study shows chick ChAM after incubation with an encircled location which indicates the agar disc positions without any extract for 48 h (negative control), the avascular zone caused by 62.5 μg/mL, and the toxic effects at 250.0 and 500.0 μg/mL extract on the ChAM. The extract disrupted mostly newly forming blood vessels without affecting the preexisting vasculature. The extract showed antiangiogenic activity, which can be further studied for understanding the mechanism for this activity and further characterization and purification of the active biomolecule responsible for this activity. Further to this, we also plan to understand the role of symbionts microorganism of these marine sponges in antiangiogenic activity.

From the studies it is concluded that AChE enzyme can be isolated from the marine sponges and further used as a template for the development of new antiangiogenic drugs. The AChE activity was maximum in *S. pachyspira* and the variability in grown samples was less. Stability of the samples was seen for almost 6 d with no major deterioration in the enzyme activity. Literature suggests marine sponges to be a good source of AChE inhibitors which also have anti-angiogenesis activity. In the present work, ChAM study showed that methanolic extract of the marine sponge *S. pachyspira* has strong antiangiogenic activity. With further purification and characterization the molecule can be used for anti tumor activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The background of the research is good and extensive. The ideas carried out in this paper are very important for both scientific knowledge of the marine organisms as a source of active molecules and for the advancement of awareness of their prospective use for human health. In particular, the anti-angiogenic potential of the AChE enzyme extracted from sponges in the care of tumors is a

very up-to-date argument.

Research frontiers

This research evaluated the effect and possible therapeutical use of active molecules extracted from marine sponges, which is very relevant for the progress of human health care.

Innovations and breakthroughs

The experiments on angiogenesis are very innovative, Thus, the results of this work might bring a contribution to new coadjuvator therapies for cancer, besides other degenerative diseases.

Applications

Medical care, pharmacology, pharmacy industry. In the past, we patented a sponge-derived molecule for new therapies, and the pharmacological industries showed interest, mainly on the possible synthetic derivatives.

Peer review

The paper deals with an important argument, cause of the multiple applications and functions of the studied enzyme. The experiments are carefully described and the figures are convincing.

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