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Screening of antiangiogenic potential of twenty two marine invertebrate extracts of phylum Mollusca from South East Coast of India

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated the anti-angiogenic property of marine invertebrates *in ovo* angiogenesis (CAM assay) model and ocular angiogenesis experimental model in rats. The activity was accessed based on the extent of inhibiting new vessel formation induced by VEGF, chemical injury and hyperoxia. Out of 22 extracts studied, *Telescopium telescopium* was found to be a potential candidate for further development for ocular angiogenesis.
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ABSTRACT

Objective: To evaluate the antiangiogenic potential of twenty two marine invertebrate species of Phylum Mollusca from south east coast of India.

Methods: Live specimens of molluscan species were collected and their methanolic extracts were evaluated for preliminary antiangiogenic activity using the *in ovo* chick chorio-allantoic membrane assay. The extracts were further evaluated for *in vivo* antiangiogenic activity using chemical cautery induced corneal neovascularization assay in rats and oxygen induced retinopathy assay in rat pups.

Results: In the chick chorio-allantoic membrane assay, four methanolic extracts of marine molluscan species *viz. Meretrix meretrix, Meretrix casta, Telescopium telescopium* and *Bursa crumena* methanolic extracts exhibited noticeable antiangiogenic activity at the tested concentration of 200 μg whereby they significantly inhibited the VEGF induced proliferation of new blood vessels. Among these four extracts, the methanolic extract of *Meretrix casta* exhibited relatively higher degree of antiangiogenic activity with an inhibitiory percentage (64.63%) of the VEGF induced neovascularization followed by the methanolic extracts of *Telescopium telescopium* (62.02%), *Bursa crumena* (60.48%) and *Meretrix meretrix* (47.01%). These four methanolic extracts were further evaluated for *in vivo* antiangiogenic activity whereby the methanolic extract of *Telescopium telescopium* exhibited most noticeable inhibition (42.58%) of the corneal neovascularization in rats in comparison to the sham treated group, and also exhibited most noticeable inhibition (31.31%) of the oxygen induced retinal neovascularization in rat pups in comparison to the hyperoxia group that was observed for considerable retinal neovascularization.

Conclusions: The significant antiangiogenic activity evinced by the extract of *Telescopium telescopium* merits further investigation for ocular neovascular diseases.

KEYWORDS

Marine invertebrate, Mollusca, Antiangiogenic, CAM, Cautery, Retinopathy

1. Introduction

Among the 34 fundamental phyla of life, 17 have been reported on land while 32 of them are found in the sea (with some overlaps) and among these, the marine organisms represent a majorly unexplored domain, having highest

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chances for the identification of compounds with higher potency and novel biological activities[1]. This has been shown in the recent past as much attention has been given to the approach of isolating novel chemical structures and compounds from marine natural products (MNPs) whereby MNPs bioprospecting has yielded a considerable number of drug candidates with potent anticancer properties with some of these possessing novel mechanisms of action[2]. Other than anticancer agents, many therapeutically successful drugs like lipid lowering agents, immuno-suppressants, antifungals, antivirals, anti-inflammatory, analgesic, anti-malarial, anti-HIV, *etc.* have been obtained from marine sources.

Marine invertebrates constitute one of the major groups of marine organisms from which a wide range of medicinal benefits have been devised in addition to the large numbers of MNPs that have been discovered till date[3]. Seafood diet from edible marine invertebrates such as molluscs has been linked with various medicinal benefits to improve human health[4]. Throughout history, molluscs have provided a wide range of human resources, including food, shells, dyes and medicines. In many cultures, shelled gastropods and bivalves are regarded as a delicacy or healthy food and they also feature in a range of traditional natural remedies[5]. In most cases, there has been no scientific research undertaken to substantiate the health benefits of molluscs. However, there is increasing interest in the bioactivity of mollusc extracts and secondary metabolites. Currently, natural products isolated from molluscs are particularly well represented in the anticancer compounds in clinical trials *e.g.* elisidepsin, a novel marine-derived cyclic peptide belonging to the Kahalalide family of compounds currently under phase II development with preliminary evidence of antitumor activity[6].

The formation of new blood vessels out of pre-existing capillaries or angiogenesis is a sequence of events that is of key importance in a broad array of physiologic and pathologic processes. In several diseases, excessive angiogenesis is a part of the pathology. These diseases include cancer (both solid and hematologic tumors[7], cardiovascular disease (atherosclerosis)[8], chronic inflammation (rheumatoid arthritis, Crohn's disease), diabetes (proliferative diabetic retinopathy)[9], neovascular wet age related macular degeneration[10], retinopathy of prematurity[11], psoriasis[12] and AIDS complications[13]. These diseases may benefit from the therapeutic inhibition of angiogenesis. A growing tumor needs an extensive network of capillaries to provide nutrients and oxygen to the body tissues. In addition, the new intratumoral blood vessels provide a way for tumor cells to enter the circulation and to metastasize to distant organs. Thus, every organ system may involve diseases in which angiogenesis is an important component. Several compounds from marine sources are under clinical trials and have been shown to possess potent angiostatic effect in the pre-clinical phases *e.g.* squalamine[14]. In recent years, many bioactive compounds have been isolated from cone snails, soft corals, sponges, sea squirts, marine worms, bryozoans, sea slug, sharks and other marine organisms and among these, shark cartilage have been recognized as an important source of bioactive compounds having antiangiogenic potential.

As marine organisms from Indian seas are considered as potential sources of bioactive molecules, this study was undertaken for the first time to explore the antiangiogenic properties of the methanolic extracts of various species of marine invertebrates from phylum Mollusca using *in ovo* chick chorio-allantoic membrane (CAM) assay. The methanolic extracts that showed noticeable *in ovo* antiangiogenic activity were further evaluated for *in vivo* antiangiogenic activity using chemical cautery induced corneal neovascularization assay in rats and oxygen induced retinopathy in rat pups.

2. Materials and methods

2.1. Drugs and chemicals

VEGF, bryostatin 1 and dolastatin 15 were purchased from Sigma-Aldrich, USA. Squalamax (natural shark squalamine extract) was purchased from Nu Gen, USA. Thalidomide was purchased from Natco pharma, Hyderabad. Silver nitrate and potassium nitrate were purchased from Qualigens, Mumbai. Bevacizumab was obtained from Genentech, USA. All the other reagents were of analytical grade and were used without further purification.

All study protocols were approved by standing Institutional Animal Ethics Committee (IAEC) of All India Institute of Medical Sciences (AIIMS), New Delhi, India. All animal experiments were done in accordance with guidelines of Association for Research in Vision and Ophthalmology.

2.2. Collection and identification of marine invertebrates

Live specimens of twenty two marine invertebrates of phylum Mollusca were collected from Cuddalore coastal

areas (latitude 11°45' N; longitude 79°45' E) of Tamil Nadu, southeast coast of India. The marine samples were brought in ice to the laboratory and stored at −20 °C. The samples were identified by Dr. M. Arumugam, marine biologist at the Centre for advanced study in marine biology, Annamalai University, Tamil Nadu. Among the twenty two molluscan species, four were identified as cephalopods, five were bivalves and thirteen were identified as gastropod species. The cephalopods were identified as *Sepiella inermis* (Sepiidae), *Sepiella brevimana* (Sepiidae), *Loligo uyii* (Loliginidae) and *Cistopus indicus* (Octopodidae). The bivalves were identified as *Perna viridis* (Mytilidae), *Meretrix meretrix* (Veneridae), *Meretrix casta* (Meretricinae), *Crassotrea madrasensis* (Ostreidae), *Anadara granosa* (Arcidae). The gastropods were identified as *Babylonia spiratta* (Babylonidae), *Rapana rapiformis* (Muricidae), *Murex virgineus* var. *ponderasa* (Muricidae), *Turritella acutangula* (Turritellidae), *Cerithidea cingulata* (Potamididae), *Telescopium telescopium* (Potmididae), *Bursa crumena* (Bursidae), *Conus betulinus* (Conidae), *Tonna dolium* (Tonnidae), *Hemifusus cochlidium* (Melongenidae), *Ficus ficus* (Ficidae), *Tudicula spirallis* (Turbinellidae) and *Conus amadis* (Conidae).

2.3. Extraction of marine invertebrates

The soft bodies of all the marine species were further processed prior to their extraction. The ink gland was removed from all cephalopods and the outer shells of the bivalves and gastropods were cut opened. All the organisms were chopped into small pieces, weighed (200 g) and macerated with methanol (500 mL) for 7 d, respectively. The extracts were filtered and concentrated to dryness using a rotary evaporator and the extracts were further stored at 4 °C until analysis.

2.4. Evaluation of antiangiogenic activity of marine molluscan species

2.4.1. Chick CAM assay

The chick CAM assay was performed as per the procedure previously standardized in our laboratory[15]. Briefly, fresh fertile white leghorn chick eggs were purchased from Kegg Farm (Gurgaon, Haryana, India) and further placed in a humidified incubator at (36±2) °C. On the third day of incubation, a hole was drilled on the egg shell and 2−3 mL of albumin was withdrawn aseptically. The hole was sealed using sterile parafilm and eggs were re−subjected for incubation. And 10 mg/mL stock solution of each extract was prepared by dissolving 10 mg extract in 100 μL DMSO and 900 μL ovalbumin. On seventh day, a suitable window was opened and VEGF (50 ng) in ovalbumin coated coverslips of 12 mm diameter (with or without test substance) were placed over the CAM. Coverslips coated only with ovalbumin, ovalbumin with VEGF, ovalbumin with VEGF and standards (thalidomide, squalamine, dolastatin 15 and bryostatin 1), ovalbumin with VEGF and methanolic extracts (200 μg) served as normal, control, positive control and test groups respectively (n=8). The windows were further sealed with sterile parafilm and reincubated for further period up till twelfth day. On Day 12, the blood vessels grown under the coverslip area were digitally photographed using a sensitive CCD camera directly attached to the computer using graphic user interface. The blood vessel areas were analysed using the aphelion developer image analysis software (Adcis, France).

2.4.2. Corneal neovascularization assay

Corneal neovascularization was induced by chemical cauterization as per the procedure previously standardized in our laboratory[15]. Briefly, one cornea of each rat was cauterized by pressing an applicator stick (with a diameter of 1.5 mm) coated with 75% silver nitrate/25% potassium nitrate on the centre of the corneas for 5 seconds while the animal was deeply anesthetized with sodium pentobarbitone. To increase the reproducibility of the injuries, the same investigator cauterized all animals. Following cauterization, the rats were randomized to eliminate potential bias in the degree of injury within the different groups. The methanol extracts of *Meretrix casta*, *Meretrix meretrix*, *Telescopium telescopium* and *Bursa crumena* were weighed (10 mg), dissolved in 100 μL DMSO and final volume was made up with normal saline to obtain a concentration of 10 mg/mL (1% w/v). Rats were randomly divided into different groups (n=6) *viz.* sham, positive control (bevacizumab 1.25 mg/mL) and methanolic extracts (1% w/v) treated. And 20 μL drop of test was applied topically to each cauterized cornea three times per day for 5 d starting from the day of cauterization. The first treatment with each medication was started approximately 30 min after cauterization. The corneal neovascularization was assessed on Day 6 after cauterization whereby the corneas of all rats were subjected for photography using CCD camera. The photographs were further subjected for quantification of corneal neovascularization using the aphelion developer image analysis software.

2.4.3. Oxygen induced retinopathy assay

Induction of retinopathy in rat pups was carried out as per the previously reported procedure[16]. Briefly, Wistar albino nursing rats with pups were placed in an incubator with 80%±2% oxygen starting at postnatal Day 7 (P7) through P12. The oxygen levels were measured with an oxygen sensor and checked atleast twice daily. On P12, animals were returned to room air. Treatment consisted of intraperitoneal injection of the test substance, once daily from P12 to P16. The methanolic extracts of *Meretrix casta*, *Meretrix meretrix*, *Telescopium telescopium* and *Bursa crumena* were weighed (10 mg), dissolved in 100 μL DMSO and final volume was made up with normal saline to obtain a concentration of 10 mg/ml (1% w/v). The rat pups were divided into different group′s *viz*. control (normoxia), control (hyperoxia), positive control (bevacizumab 1.25 mg/mL) and test (methanolic extracts 100 mg/kg) groups respectively. The fundus imaging of all the rat pups was carried out using Micron III retinal imaging microscope (Phoenix Research Laboratories, USA). The retinal images were further subjected for quantification using the aphelion developer image analysis software.

2.5. Statistical analysis

All the Data presented as mean±SEM. The statistical analysis was performed using unpaired student–*t* test using Sigma plot version 11 (Systat, California) and $P<0.05$ was considered as significant.

3. Results

3.1. CAM Assay

The pro-angiogenic growth factor VEGF at the tested concentrations of 50 ng induced noticeable degree of proliferation of new blood vessels that was observed to be significantly ($P\leq0.001$) higher than the normal (without VEGF) group. Thalidomide was used as a positive control for the study that at a concentration of 10 μg showed 70.08% inhibition of VEGF induced neovascularization. A known antiangiogenic agent of marine origin *i.e.* squalamine was also used as a standard that exhibited 75.87% inhibition of VEGF induced proliferation of new blood vessels at a concentration of 6 μg. Anticancer marine isolates *viz*. dolastatin 15 and bryostatin 1 were also used as controls whereby both the drugs profoundly inhibited the VEGF induced proliferation of new blood vessels though the degree of inhibition varied with the two. Dolastatin 15 at a concentration of 1 000 ng exhibited 64.44% inhibition whereas Bryostatin 1 at the same concentration exhibited relatively higher degree of inhibition (75.68%) of the VEGF induced angiogenesis .

Among the twenty two methanolic extracts of the marine invertebrates, four extracts *viz*. *Meretrix meretrix, Meretrix casta, Telescopium telescopium* and *Bursa crumena* methanolic extracts were observed for noticeable antiangiogenic activity at the tested concentration of 200 μg thereby signifying their anti-VEGF potential (Figure 1) and were further evaluated for *in vivo* antiangiogenic activity. Among these four methanolic extracts, the *Meretrix casta* extract exhibited noticeably higher antiangiogenic activity with an inhibition of 64.63% of the VEGF induced neovascularization. The methanolic extracts of *Telescopium telescopium* and *Bursa crumena* showed comparable effects as they were observed for 62.02% and 60.48% inhibition of new blood vessels formation while the methanolic extract of *Meretrix meretrix* showed only 47.01% inhibition of neovascularization in comparison to the VEGF control group. The total area of blood vessels in the coverslip area for all the groups was quantified using the image analysis software (Table 1).

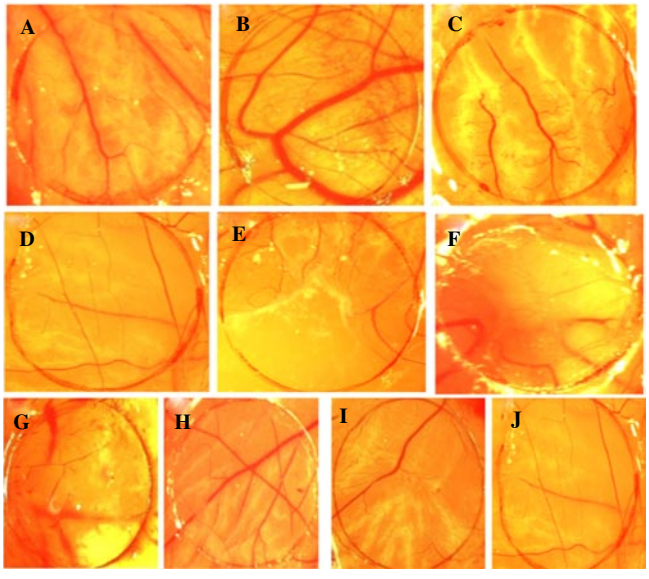

Figure 1. Effect of methanolic extracts of marine invertebrate species on the chick CAM observed on Day 12 of incubation. Photographs showing blood vessels on the CAM for different groups.

A. Normal. B. Treated with VEGF 50 ng. C. Treated with Thalidomide 10 μg along with VEGF 50 ng. D. Treated with Squalamine 6 μg along with VEGF 50 ng. E. Treated with Dolastatin 1 μg along with VEGF 50 ng. F. Treated with Bryostatin 1 μg along with VEGF 50 ng. G. Treated with Meretrix casta methanolic extract 200 μg along with VEGF 50 ng. H. Treated with *Meretrix meretrix* methanolic extract 200 μg along with VEGF 50 ng. I. Treated with *Telescopium telescopium* methanolic extract 200 μg along with VEGF 50 ng. J. Treated with *Bursa crumena* methanolic extract 200 μg along with VEGF 50 ng.

Table 1
Representation of vessel area per coverslip in CAM assay.

Group/Extract	Vessel Area/Coverslip (mm×mm)
Normal	38.916±0.375*
Control (VEGF 50 ng)	91.019±0.184*
Thalidomide 10 μg+VEGF 50 ng	27.233±0.352*
Bryostatin 1 μg+VEGF 50 ng	22.145±0.756*
Dolastatin 1 μg+VEGF 50 ng	32.393±0.611*
Squalamine 6 μg+VEGF 50 ng	21.963±0.460*
Sepiella inermis+VEGF 50 ng	90.539±0.199
Perna viridis+VEGF 50 ng	90.013±0.458
Meretrix meretrix+VEGF 50 ng	48.222±0.961*
Meretrix casta+VEGF 50 ng	32.192±0.428*
Babylonia spiratta+VEGF 50 ng	57.569±0.485*
Loligo uyii+VEGF 50 ng	79.845±0.703
Crassotrea madrasensis+VEGF 50 ng	90.221±0.458
Cistopus indicus+VEGF 50 ng	81.754±0.589
Sepiella brevimana+VEGF 50 ng	90.959±0.444
Anadara granosa+VEGF 50 ng	90.343±0.366
Rapana rapiformis+VEGF 50 ng	90.305±0.321
Murex virgineus var. *ponderasa*+VEGF 50 ng	89.320±0.750
Turetella acutangula+VEGF 50 ng	90.529±0.400
Cerithidea cingulatta+VEGF 50 ng	90.770±0.401
Telescopium telescopium+VEGF 50 ng	34.564±0.486*
Bursa crumena+VEGF 50 ng	35.963±0.972*
Conus betulinus+VEGF 50 ng	77.096±0.440
Tonna dolium+VEGF 50 ng	89.044±0.969
Hemifusus cochlidium+VEGF 50 ng	90.282±0.278
Ficus ficus+VEGF 50 ng	55.884±0.527*
Tudicula spirallis+VEGF 50 ng	90.147±0.480
Conus amadis+VEGF 50 ng	90.614±0.332
10% DMSO in ovalbumin+VEGF 50ng	90.806±0.225

Data presented as mean±SEM (n=8). *P≤0.001 *vs* Control Group.

3.2. Cautery induced corneal neovascularization

There was no corneal neovascularization observed in the normal (uncauterized) corneas. Among all the four tested extracts, the methanolic extract of *Telescopium telescopium* exhibited most noticeable inhibition (42.58%) of the chemical cautery induced corneal neovascularization in rats (Figure 2) in comparison to the sham treated group that was observed for considerable neovascularization in the cauterized corneas of rat eyes. Comparatively, bevacizumab (positive control) showed significant (P≤0.001) inhibition (70.05%) of the cautery induced corneal neovascularization. The methanolic extract of *Bursa crumena* showed 26.74% inhibition of the corneal neovascularization. The methanolic extracts of *Meretrix* species *viz*. Meretrix casta and *Meretrix meretrix* were found to offer only 19.7% and 12.14% inhibition respectively in this model (Table 2).

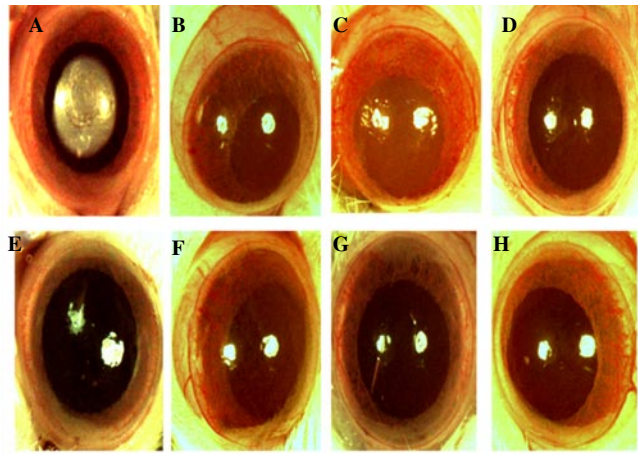

Figure 2. Effect of methanolic extracts of marine invertebrate species in cautery induced corneal neovascularization in rats. Photographs showing rat corneas for different groups.
A. Cauterized cornea. B. Normal cornea. C. Sham treated cornea. D. Bevacizumab 1.25 mg/mL treated cornea. E. *Telescopium telescopium* methanolic extract (1% w/v) treated cornea. E. *Bursa crumena* methanolic extract (1% w/v) treated cornea. E. *Meretrix casta* methanolic extract (1% w/v) treated cornea. E. *Meretrix meretrix* methanolic extract (1% w/v) treated cornea.

Table 2
Vessel area in the cauterized corneas of different groups.

Group/Extract	Area of corneal blood vessels (mm×mm)
Sham	17.05±0.499
Bevacizumab	5.11±0.283*
Meretrix meretrix	14.98±0.464
Meretrix casta	13.69±0.302*
Telescopium telescopium	9.79±0.345*
Bursa crumena	12.49±1.168*
10% DMSO in normal saline	17.55±0.316

Data presented as mean±SEM (n=6). *P≤0.05 *vs* Sham treatment.

3.3. Oxygen induced retinopathy assay

The hyperoxia (80% oxygen) treated control group rat pups showed significant (P≤0.001) retinal neovascularization in comparison to the normoxia treated rat pups which were kept at ambient air (21% oxygen). The positive control bevacizumab exhibited significant (P≤0.001) inhibition of the oxygen induced retinal neovascularization in rat pups in comparison to the hyperoxia group. Among all the four tested methanolic extracts of marine invertebrate's *viz*. *Meretrix meretrix*, *Meretrix casta*, *Telescopium telescopium* and *Bursa crumena*, the extract of *Telescopium telescopium* exhibited most noticeable inhibition (31.31%) of the oxygen induced retinal neovascularization in rat pups (Figure 3) in comparison to the hyperoxia group whereas the remaining extracts of *Bursa crumena*, *Meretrix casta* and *Meretrix meretrix* were observed for only 15.53%, 14.78% and 8.64% inhibition of the oxygen induced retinal neovascularization (Table 3).

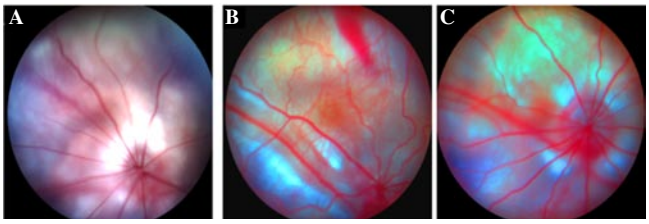

Figure 3. Effect of methanolic extract of *Telescopium telescopium* in oxygen induced retinal neovascularization in rat pups. Photographs showing rat fundus for different groups.
A. Normoxia group. B. Hyperoxia group. C. *Telescopium telescopium* methanolic extract (100 mg/kg) treated group.

Table 3
Quatification of oxygen induced retinal neovascularization.

Group/Extract	Area of corneal blood vessels (mm×mm)
Normoxia	4.13±0.16*
Control (Hyperoxia)	11.02±0.25
Bevacizumab	5.19±0.72*
Meretrix meretrix	10.07±0.17*
Meretrix casta	9.39±0.13*
Telescopium telescopium	7.56±0.08*
Bursa crumena	9.31±0.11*
10% DMSO in normal saline	10.98±0.07

Data presented as mean±SEM (n=6). *P≤0.001 *vs* Control Group.

4. Discussion

The quest of antiangiogenic compounds for the treatment of neovascular diseases has yielded an array of extracts of marine species particularly the invertebrates that bear potent angiostatic effects *e.g.* methanolic extract of the feather star, Lamprometra palmata palmate, whole body extract of marine gastropod *Euchelus asper*, *etc*[17,18]. These extracts of marine invertebrates are sources of novel secondary metabolites of unprecedented molecular structures and activities. The phylum Mollusca represents a majorly unexplored domain in the marine ecosystem as there are up to 150 000 molluscan species and contrary to their vast numbers and relative accessibility, not many of these species have been investigated for their pharmacological potential. Therefore, the present study was designed to evaluate the antiangiogenic potential of this majorly unexplored class of marine species, particularly phylum Mollusca that has been a potential source of several anticancer compounds such as dolastatins, Aplyronine A, Kahalalide F and Jorunnamycins A-C[19].

Angiogenesis, the physiological process involving the growth of new blood vessels from pre-existing ones, is essential for organ growth and repair. Angiogenesis involves a series of co-ordinated events such as endothelial cell proliferation and migration, capillary tube formation, extracellular matrix degeneration and remodeling[20]. The process of angiogenesis is tightly regulated by the balance between angiogenesis stimulators and inhibitors. However, the imbalance in angiogenesis contributes to numerous pathologies that may play a pivotal role in tumor growth and metastasis. In several ocular neovascular diseases such as age related macular degeneration, diabetic retinopathy and retinopathy of prematurity, excessive angiogenesis occurs when diseased cells produce abnormally large amounts of angiogenesis factors *e.g.* VEGF, overwhelming the effects of natural angiogenesis inhibitors such as angiostatin. Antiangiogenic therapies, which are aimed at suppressing new blood vessel growth, are being developed for treating these chronic ocular diseases that are the leading causes of blindness in the infants, adults of working age and the elderly in the developed world[21]. Today, over 20 angiogenic growth factors and over 300 antiangiogenic molecules from natural and synthetic sources, targeting different signaling pathways are being tested for their anticancer properties at preclinical and clinical stages and among these the anti-VEGF therapies are being looked forward for the treatment of pathological ocular neovascularization[22,23].

The chick CAM assay is a preliminary *in ovo* assay technique and since the CAM has a very dense capillary network, it is commonly employed for evaluating neovascularization and its inhibition in response to different angiogenic stimulators[24]. An important growth factor in angiogenesis is VEGF that has been applied on the CAM from Day 7 to Day 12 for the induction of neovascularization. In the CAM of embryos of this age, VEGF induces vascular growth in the region of the capillaries and also in the pre- and post-capillary vessels by sprouting angiogenesis. VEGF stimulates the cells to produce matrix metalloproteinases, which degrade the basement membrane and surrounding extracellular matrix. As a result, endothelial cells proliferate and migrate towards the interstitium, where they start sprouting. Subsequently, the cells proliferate and migrate towards the newly formed sprouts and mature by forming single cell layer around the sprout[25]. Antiangiogenic drugs prevent the binding of VEGF with its receptors on the surface of the endothelial cells. Owing to its central role in promoting tumor growth, VEGF has now become a key therapeutic target and its functions can be blocked at different levels of the signaling pathways. Therefore, in this study VEGF was used as an angiogenic stimulator in CAM assay at a concentration of 50 ng for the stimulation of controlled angiogenesis within the coverslip area of 113.14 mm^2. Similar concentration was also used for the induction of neovascularization in the chick embryo's by Lingaraju and coworkers[26].

Thalidomide was used as a positive control for the CAM assay as its an known inhibitor of angiogenesis[27]. The effect of thalidomide on growing vasculature in chick CAM without any inducer of angiogenesis has been demonstrated previously whereby it has been reported that the implantation

of 0.5% CMC pellets containing thalidomide did not exhibited any noticeable antiangiogenic effect in the embryonic vasculature[28]. However, further studies reported that 100 μg/mL thalidomide disc noticeably blocked nitric-oxide mediated angiogenesis in an ex-ovo chick CAM assay[29]. In contrast, the present study directly evaluated the effect of thalidomide on the VEGF induced angiogenesis in the chick chorioallantoic membrane assay for the first time. Thalidomide at a concentration of 10 μg was observed to perturb the embryonic vasculature that was induced by the action VEGF. Squalamine, an aminosterol antibiotic from the liver of the dogfish shark, *Squalus acanthus*, is a proven antiangiogenic compound of marine origin that is undergoing clinical trials for the treatment of age related macular degeneration. Therefore, it was included in the present study for an effective correlation of newer extracts. Squalamine extract at a concentration of 6 μg was observed for noticeable inhibition of the embryonic vasculature induced on the CAM by VEGF. Two anticancer marine isolates *viz*. dolastatin 15 and bryostatin 1 were also used as controls in the CAM assay. Dolastatins are unique natural peptides derived from the marine mollusca Dolabella auricularia and dolastatin 15 is a small peptide from the class of dolastatins that have been identified as an inhibitor of microtubule assembly that induces mitotic arrest and apoptosis of human multiple myeloma cells[30]. The results showed that dolastatin 15 produced noticeable inhibition (64.44%) of VEGF induced angiogenesis in the CAM assay at a concentration of 1 μg. Bryostatin 1 is a macrolactone isolated from the marine bryozoan Bugula neritina and is a protein kinase C activator that has been reported to inhibit leukemic growth in vitro via cell cycle arrest at G2 (as well as G1)[31]. The results showed that bryostatin showed a maximum of 75.68% inhibition at a concentration of 1 μg which was comparatively higher to the inhibition observed with dolastatin 15 at the same concentration indicating higher potency of bryostatin 1.

Among all the twenty two marine invertebrate extracts evaluated for their antiangiogenic potential, the methanolic extracts of *Meretrix meretrix*, *Meretrix casta*, *Telescopium telescopium* and *Bursa crumena* exhibited significant ($P \leq 0.001$) anti-angiogenic activity in comparison to the VEGF control group. However, among all the four extracts, an appreciable antiangiogenic activity was observed with the methanolic extract of *Meretrix casta* followed by *Telescopium telescopium*, *Bursa crumena* and *Meretrix meretrix* extracts. Interestingly, the activity of all the four methanolic extracts was found have dose dependent positive correlation in antiangiogenic activity. These obervations make them worthy of subjecting for further *in vivo* studies.

Corneal neovascularization is a consequence of infection, inflammation, trauma and toxic or degenerative corneal disorders. Corneal NV may cause oedema, scar formation or lipid deposition leading to significant visual impairment[32]. Corneal neovascularization appears to be controlled by two opposing mechanisms: angiogenic stimulators such as VEGF and angiogenic inhibitors such as angiostatin. Under normal conditions, the balance is toward the endogenous angiogenic inhibitors, keeping the cornea avascular. An insult to the cornea, may enhance the production of angiogenic stimulators, disturing the balance and resulting in capillary endothelial cell proliferation and neovascularization. In the present study, 75% silver nitrate/25% potassium nitrate applicator sticks were used for the purpose of cauterization of the rat corneas. As the silver nitrate contacts with water (in the blood) the compound goes into solution forming nitric acid which is subsequently responsible for the cautery effect. The combination of silver nitrate with potassium nitrate toughens silver nitrate so that it can be applied quickly and conveniently to the precise area to be cauterized without spread to the adjacent healthy tissues.

VEGF is a potent and highly selective mitogen for vascular endothelial cells and substantial evidences indicate that VEGF and its receptors play an important role in the pathophysiology of corneal NV and are upregulated in corneal angiogenesis thereby playing a key role in the pathogenesis of corneal neovessels[33]. Pharmacological treatment of corneal neovascularization using angiogenic inhibitors has evolved a new way to manage corneal neovascularization. Lately, the success of monoclonal antibodies against VEGF such as bevacizumab in the treatment of retinal and choriodal neovascularization has encouraged the use of these antibodies to treat corneal neovascularization and this is evident from the pre-clinical studies that reported topically administered bevacizumab significantly reduces corneal NV in rats[34]. Bevacizumab was used as a positive control for the present study that showed significant inhibition of the cautery induced corneal neovascularization in rats in comparison to the sham treated group.

The methanolic extracts of *Telescopium telescopium*, Bursa crumena, *Meretrix meretrix* and *Meretrix casta* that exhibited noticeable anti-angiogenic activity in the CAM assay were further evaluated for their *in vivo* antiangiogenic potential using silver nitrate cautery induced corneal neovascularization assay in rats. For the topical application in the eye, the stock solution (10 mg/mL) of the extracts was prepared by dissolving 10 mg of each extract in 10% DMSO and volume made up with normal saline. The osmolarity of the four solutions of methanolic extracts of *Meretrix meretrix*, *Meretrix casta*, *Telescopium telescopium* and *Bursa crumena* were measured using osmometer (μ OsmetteTM 5004, Precision Systems, USA) and was found to be 410, 416, 413 and 405 mOsm, respectively.

The methanolic extract of *Telescopium telescopium* exhibited most noticeable inhibition of the chemical cautery induced corneal neovascularization in rats which was highest among all the tested extracts but 1.5 folds lower than the positive control *i.e.* bevacizumab. The significant anti-angiogenic activity observed for the extract may be due to the anti-VEGF activity as observed for the extract in the CAM assay. However, as the biological system serves as an epicentre for several growth mediators of angiogenesis other than VEGF, therefore a possibility of inhibition of other mediators cannot be ruled out, thus emphasizing for further insght into the mechanisms of actions involved for the extract. The methanol extracts of other three molluscan species *viz*. *Bursa crumena*, *Meretrix casta* and *Meretrix meretrix* showed only slight antiangiogenic activity as they were observed for only 26.74%, 19.7% and 12.14% inhibition of the cautery induced corneal neovascularization in rats. In this study, the protection offered by the extracts were found to be significant but relatively low as compared to bevacizumab. This might be due to the presence of lower concentrations of active principle in the prepared extracts. Furthermore, due to non-solubility of the entrire extract, this study used 10% DMSO for solubilizing the extract in saline to enable its topical instillation. DMSO at 10% can cause ocular surface irritation and cause lacrimation which can clear precorneal tear drug concentraiton in a faster rate. Although angiogenesis found in the vehicle treted control was undifferentiatable from the sham treated animal, effect of DMSO interfering the marine isolates for their protective action can not be ruled out.

The functional relationship between inflammation and tumors is not new as the role and involvement of angiogenesis for the growth of tumors has been widely recognized and antiangiogenic drugs with inherent anti-inflammatory potential seem likely to be biologically plausible for the treatment of inflammation dependent pathological ocular angiogenesis primarily observed in ocular tumors and corneal neovascularization[35]. Inflammation-dependent angiogenesis seems to be a central force in tumor growth[36], a concept supported by the observation that the use of classical anti-inflammatory drugs, such as non steroidal anti inflammatory drugs (NSAIDs), leads to the inhibition of ocular neovascularization *e.g.* nepafenac[37]. The mechanisms of inflammatory angiogenesis provide new approaches to cure or prevent angiogenesis occurring in cases of ocular tumors and pathological corneal neovascularization. In the present study, the methanolic extract of *Telescopium telescopium*, that exhibited noticeable inhibition of the chemical cautery induced corneal neovascularization in the rats, signify its potential for further development as an novel antiangiogenic therapy for the treatment of inflammation dependent pathological ocular angiogenesis.

In the retina, hypoxia may occur as a result of vascular disruption caused by various pathologies, such as hyperglycaemia in diabetes, thrombosis in vein occlusions or developmental delays in retinopathy of prematurity. Normally, hypoxia is a key driving force inducing a vascular response, whereby insufficiently perfused tissue is revascularized by the sprouting of new capillaries from pre-existing vessels. In theory, hypoxia in the eye should therefore result in compensatory revascularization to replenish vessel starved areas. However, for reasons not well understood, the revascularization is sometimes not successful, leading to abnormal vessels so called 'neovascularization'-instead of healthy, new capillaries. This is a major vision-threatening complication in many ischemic retinopathies because the abnormal blood vessels leak, cause edema and exert tractional forces, causing in worst case scenarios retinal detachment.

The rat model of oxygen-induced retinopathy has been widely used as an animal model of the human form of this disease, retinopathy of prematurity. The oxygen-induced retinopathy model in rat pups provides an excellent model system for evaluating abnormal angiogenesis in the retina that includes reproducible proliferative retinal neovascularization. In contrast with other animal models that have been used to study the pathophysiology of this disease, the neonatal rats offer the advantage of a highly immature retina at birth that is comparable to that of a 24-26 week old human embryo. As in humans, exposure of the rat pups to postnatal hyperoxia from P7 to P12 causes severe vasoconstriction and vaso-obliteration, followed by an abnormal proliferation of retinal vessels upon return to room air due to the oxidative stress[38].

Among all the four extracts of molluscan species, the methanolic extract of *Telescopium telescopium* exhibited most noticeable inhibition of the oxygen induced retinal neovascularization in rat pups. The significant ($P \leq 0.001$) antiangiogenic activity observed for the extract may be due to its anti-VEGF potential as VEGF has been implicated as one of the key underlying factors in the pathogenesis of retinal neovascularization[39]. The methanolic extracts of other three molluscan species *viz*. *Bursa crumena*, *Meretrix casta* and *Meretrix meretrix* exhibited only slight antiangiogenic activity as they were observed for 15.53%, 14.78% and 8.64% inhibition of the oxygen induced retinal neovascularization in rat pups. In this study, the protections offered by the extracts were found to be significant but the inhibitory percentages were lower that might be due to the presence of lower concentrations of active principles in the prepared extracts.

This is the first report on the antiangiogenic potential of twenty two marine invertebrate species from phylum mollusca from the Indian oceans. The present study revealed *in ovo* antiangiogenic potential of *Meretrix casta*, *Telescopium*

telescopium, *Bursa crumena* and *Meretrix meretrix* extracts and further evaluated them for *in vivo* antiangiogenic potential. The noticeable inhibition of the cautery induced corneal neovascularization and oxygen induced retinal neovascularization evinced by the methanolic extract of *Telescopium telescopium* merits further investigation for ocular neovascular diseases. Detailed phytochemical examination of the methanolic extract of *Telescopium telescopium* to isolate the putative active principles responsible for the observed activity is in progress in our laboratory.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Angiogenesis is a complex process and dys-regulation of which leads to various pathologies including ocular angiogenesis leading to vision loss. The currently available state-of-the art anti-angiogenic therapies targets the VEGF pathway which is a key player in angiogenesis. However, the benefits of current anti-angiogenic agnets are verymuch limited with serious side effects. Therefore, there is a need to explore newer anti-angiogenit agents from marine source because of its special mode of action.

Research frontiers

The present study investigated the effect of methanolic extract of marine invertebrates from Indian Ocean on experimentally induced angiogenesis both ex vivo and *in vivo* and assessed its anti-angiogenic property for its future development as a potential candidate for ocular angiogenesis

Related reports

Vascular endothelial growth factor (VEGF) plays a major role in angiogenesis. The secondary metabolites obtained from marine organisms gaining momentum in anti-tumor drug discovery process in the recent past and they are reported to have anti-angiogenic properties.

Innovations and breakthroughs

Marine invertebrates constitute one of the major groups of marine organisms and many potential anti-angiogenic compounds have isolated from them for human use. In the present study, authors have demonstrated the anti-angiogenic activity of methanolic extracts of phylum mollusca in CAM assay, corneal neovascularization assay and oxygen induced retinopathy assay

Applications

Drugs derived from marine mollusks are reported to be very safe for human use. The present study support and suggest the use of this marine organism as a resource to isolate compounds against ocular angiogenesis.

Peer review

This is a valuable research work in which authors have demonstrated the anti-angiogenic property of marine invertebrates *in ovo* angiogenesis (CAM assay) model and ocular angiogenesis experimental model in rats. The activity was accessed based on the extent of inhibiting new vessel formation induced by VEGF, chemical injury and hyperoxia. Out of 22 extracts studied, *Telescopium telescopium* was found to be a potential candidate for further development for ocular angiogenesis.

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