Marine Algal Secondary Metabolites Promising Anti-Angiogenesis Factor against Retinal Neovascularization in CAM Model

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Abstract
Retinal angiogenesis is an angle of new blood vessels on retinal surface. This neovascularization condition within the eye contributes to visual loss. Commonest cause of this condition includes diabetes, retinopathy of prematurity, retinal vein occlusion, etc. A variety of endothelial cell growth factors have been identified as a responsible factor and previous studies report that marine metabolites are promising molecules against retinal angiogenesis. Based on the background information collected, the present study focused to insight the anti-angiogenesis effect of metabolites present in marine algae. Findings of CAM assay suggested that the extract obtained from the marine algae Dictayota dichotoma are effective against angiogenesis.

Keywords: Retinal angiogenesis, marine algae, CAM model, anti-angiogenesis

INTRODUCTION
Retinopathies are common causes of blindness in all age groups worldwide; to notify, age-related macular degeneration in the elderly, diabetic retinopathy in the middle aged, and retinopathy of prematurity and retinoblastoma in children. The pathogenesis of all these diseases is the retinal angiogenesis and the mediators are growth factors, vascular endothelial cells, extracellular matrix molecules, chemokines and cell signaling molecules [1]. Also the molecular mechanism involves vascular endothelial cell activation, proteolytic endothelial basement membrane degradation, extracellular matrix degradation, endothelial cell migration, vascular proliferation, formation of tight junctions, recruitment of pericytes, and deposition of new basement membrane, closing off the newly formed arteriovenous collateral vessels [2–4]. In retina, the opacification of the cornea or permanent deleterious changes to the neuronal architecture due to angiogenesis lead to irreversible visual impairment. This necessitates early and aggressive management of ocular neovascular conditions [5].

Over half of the commercial drugs are either extracted from natural sources or produced by synthesis using natural products as templates or starting materials. Ocean has the vast biodiversity and marine algae are a source of fiber, minerals, antioxidants, vitamins, pigments, steroids, lectins, halogenated compounds, polyketides, polysaccharides, mycosporine-like amino acids, proteins, polyunsaturated fatty acids and other lipids [6]. These compounds have antimicrobial, antiviral, antitumor, antioxidative, cardioprotective (antihypertensive, antiatherosclerotic and anticoagulant), immunomodulatory, analgesic, anxiolytic anti-diabetic, appetite suppressing and neuroprotective activities that have attracted the attention of the pharmaceutical industry, which attempts to design them for use in the treatment or prevention of various diseases [7]. The growing interest in marine-derived medicinal compounds would significantly expedite the exploration of significant pharmacological applications [8]. The previous knowledge in the field of retinal angiogenesis and anti-angiogenesis properties of marine compounds provide the promising target based drug identification. Based on this, in the present study the marine algae were collected and examined.
MATERIALS AND METHODS

The marine algae were collected; required extracts were obtained using specific solvents and the anti-angiogenesis test was done with all the extracts. The experimental details are explained in subsequent sections and graphically represented in Figure 1.

Sample Collection

The marine algae *Bifurcaria bifurcata* and *Dictayota dichotoma* were collected from Manapad coastal region (Tuticorin Dist.) of Tamil Nadu, India. Collected samples were kept in an ice box and immediately transported to the laboratory. Samples were washed thoroughly with tap water to remove the soil, salt and other debris on the surface of the sample and identified the species by marine species international portal (http://species-identification.org/) [9].

Crude Compound Extraction

The shade dried (10 days) sea weeds were powdered in an electric grinder and kept in the airtight plastic containers at room temperature for further analysis. The extract containing the putative anti-angiogenic compounds 4a-acetyldictyodial (BB001), (S)-12-hydroxygeranylgeraniol (BB004), pachydictyl A (BD031) and isopachydictyl A (BD032) were obtained by soaking the powders for 48 h in the appropriate solvents: chloroform: methanol (1:1), diethyl ether, petroleum ether: ether (1:1) and ethanol respectively. The fractions were subjected to evaporation of solvents after sieving through filter paper [10, 11].

Anti-angiogenesis Model

Fertilized chicken eggs (6 days old; weight 50 ± 2 g) were obtained from Tamil Nadu Veterinary and Animal Science University, Chennai and maintained at 37.5°C. Based on protocol of chick chorioallantoic membrane (CAM) method, 2 to 3 ml of albumin was withdrawn, using a gauge needle through the large blunt edge of the egg in order to minimize adhesion of the shell membrane and a window was opened in the egg shell. After exposing the vascular zone of the CAM, a sterilized filter-paper disk is employed, which is used as a carrier for being loaded with various concentrations (0.25, 0.5, 0.75 and 1 mg/ml) of marine algae extracts [12]. The marketed drug was used as a standard, and for control CAM was treated with PBS. PBS was prepared by diluting 1M of K2HPO4 (80.2 ml) and 1M of KH2PO4 (19.8 ml) to DMSO, pH was adjusted to 7.4 and autoclaved. Treated CAM was photographed at regular intervals of 0, 2, 4 and 6 h and quantified by counting the number of blood vessel branch points at the area of treatment by AngioTool software [13].
Kinetics of Anti-angiogenesis Effects of Drug Leads
The kinetics of effect of drug leads on CAM model with respect to time was correlated by the first order differential Eq. (1):

\[ \frac{dS}{dt} = KS \]  

(1)

Where, \( S \) is the substrate concentration (mg/l), \( KS \) is the first order rate constant (h\(^{-1}\)) and \( t \) is the incubation time (h). Further, Haldane’s model (Eq. (2)) was applied for determination of the kinetic parameters that responds to drug in the model system even at inhibitory levels of substrate (drug).

\[ \mu = \frac{\mu_{\text{max}} S}{K_i + S + \frac{S^2}{K_i}} \]  

(2)

\( \mu \)

Where, \( K_i \) is the inhibition coefficient (mg/l). The biokinetic parameters in this model were estimated by the Lineweaver-Burk plot (1/\( \mu \) vs. 1/S) [14].

RESULTS
Sample Collection
The marine algae *Bifurcaria bifurcata* and *Dictayota dichotoma* (Figure 2) were morphologically identified and handpicked from substratum in Manapad coastal region (Tuticorin Dist.) of Tamil Nadu, India.

Crude Compound Extraction
The dry weight of shade dried *Bifurcaria bifurcata* and *Dictayota dichotoma* results 6.66 and 8% of wet weight. The crude extraction were obtained by the solvent combinations: Chloroform: Methonol (1:1), Diethyl ether, Petroleum ether: Ether (1:1) and Ethanol. The extraction yielded 0.872, 3.499, 2.764 and 5.071 g of compounds respectively and contained the putative anti-angiogenesis compounds 4a-acetyldictyodial, (S)-12-hydroxygeranyl geraniol, pachydictyol A and isopachydictyol A respectively in each extract (Figure 3). The percentages of solvent fraction extracted are given in Table 1.

![Fig. 2: Algae Collected from Manapad Coastal Region, Tamil Nadu.](image)

**Table 1:** Compounds Extracted from Collected Algae.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compound (Drug Lead)</th>
<th>SWMD ID</th>
<th>Algae</th>
<th>Solvents</th>
<th>Solvent Extract (% of gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4a-acetyldictyodial</td>
<td>BB001</td>
<td><em>Bifurcaria bifurcata</em></td>
<td>Chloroform: Methonol::1:1</td>
<td>7.77</td>
</tr>
<tr>
<td>2</td>
<td>(S)-12-hydroxygeranyl geraniol</td>
<td>BB004</td>
<td><em>Bifurcaria bifurcata</em></td>
<td>Diethyl ether</td>
<td>1.74</td>
</tr>
<tr>
<td>3</td>
<td>Pachydictyol A</td>
<td>BD031</td>
<td><em>Dictoyota dichotoma</em></td>
<td>Petroleum ether: Ether::1:1</td>
<td>2.30</td>
</tr>
<tr>
<td>4</td>
<td>Isopachydictyol A</td>
<td>BD032</td>
<td><em>Dictoyota dichotoma</em></td>
<td>Ethanol</td>
<td>4.53</td>
</tr>
</tbody>
</table>
Fig. 3: Compounds (a) 4a-acetyldictyodial, (b) (S)-12-hydroxygeranyl geraniol, (c) Pachydictyol A and (d) Isopachydictyol A Present in the Crude Extracts of Marine Algae. (Source: ChemSpider, Seaweed Metabolite Database).

Fig. 4: Images of CAM Assay.
BB001 (0.25 mg/ml) at 0 h (a1) and at 6 h (a2); BB001 (0.5 mg/ml) at 0 h (b1) and at 6 h (b2); BB001 (0.75 mg/ml) at 0 h (c1) and at 6 h (c2); BB001 (1 mg/ml) at 0 h (d1) and at 6 h (d2); BB004 (0.25 mg/ml) at 0 h (e1) and at 6 h (e2); BB004 (0.5 mg/ml) at 0 h (f1) and at 6 h (f2); BB004 (0.75 mg/ml) at 0 h (g1) and at 6 h (g2); BB004 (1 mg/ml) at 0 h (h1) and at 6 h (h2); BD031 (0.25 mg/ml) at 0 h (i1) and at 6 h (i2); BD031 (0.5 mg/ml) at 0 h (j1) and at 6 h (j2); BD031 (0.75 mg/ml) at 0 h (k1) and at 6 h (k2); BD031 (1 mg/ml) at 0 h (l1) and at 6 h (l2); BD032 (0.25 mg/ml) at 0 h (m1) and at 6 h (m2); BD032 (0.5 mg/ml) at 0 h (n1) and at 6 h (n2); BD032 (0.75 mg/ml) at 0 h (o1) and at 6 h (o2); BD032 (1 mg/ml) at 0 h (p1) and at 6 h (p2); Standard (0.5 mg/ml) at 0 h (q1) and at 6 h (q2); Phosphate Buffer at 0 h (r1) and at 6 h (r2).
Anti-angiogenesis Model
The drug leads identified were tested for CAM anti-angiogenesis model with 6 days fertilized eggs. The analysis of branch points showed that, the compound BD032 at 0.5 mg/ml showed highest reduction of blood vessels (88.37%), which is 3.52% more than the standard drug (Figure 4). This is followed by BD031 at 0.5 mg/ml which reduces 81.82% of blood vessels after 6 h. The minimum effect (31.58%) was observed in BB004 at 0.25 mg/ml (Figure 5).

Kinetics of Anti-Angiogenesis Effects of Drug Leads
The graphical representation of Haldane’s kinetic model illustrates that the effects of drug leads BB004, BD031 and BD032 increases along with concentration up to a certain maximum value and the rate decreases (Figure 6). In case of BB001, the effect increases with respect to increasing concentration of the dose. It was obvious that the effect of the compounds BD031 and BD032 which were extracted from Dictayota

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**Fig. 5:** Effects of Seaweed Extracts on Anti-Angiogenesis Model (CAM Assay).

**Fig. 6:** Dose-Response Curve of Drug Leads against Angiogenesis.
dichotoma were higher than BB001 and BB004 which were extracted from Bifurcaria bifurcate.

DISCUSSION
In solvent extraction from marine algae, the solvents with higher polarity (chloroform: methanol and ethanol) produce more solvent fractions when compared to low polarity solvents as expected. In CAM assay, the increasing of dosage concentration reduces the blood vessels; that implies the efficiency of marine algae against neovascularization. Also, it was found that the extracts obtained from Dictoyota dichotoma showed higher effect than the ones extracted from Bifurcaria bifurcata. According to the potential relevance of dose-response curve proposed by Reynolds, the bell-shaped nature of the response of BD031 and BB004 are characterized by dose stimulation, followed by loss of effects at higher dose [15]. The compound BB001 may even have stimulating effect at higher doses, which leads to J-shaped dose-response and U-shaped nature of BB004 showed that at low concentration, it has higher inhibitory effect.

CONCLUSION AND RECOMMENDATION
The results of this present study, correlated with the results of in silico studies that the inhibition constant of BD031 (17.86 uM), BD032 (13.8 uM) is less than BB001 (4.19 mM) and BB004 (2.57 mM) [16]. As higher concentration is required for (S)-12-hydroxygeranyl geraniol (BB004) and 4a-acetyldictyodial (BB001) to inhibit PAI1 target protein, they showed less effect at the same concentration used for the compounds pachydictyol A (BD031) and isopachydictyol A (BD032). The results concluded that, the extract which containing (S)-12-hydroxygeranyl geranial and 4a-acetyldictyodial (0.5 mg/ml) obtained from the marine algae Dictoyota dichotoma are effective against angiogenesis in CAM model. Further, it is recommended for the in vitro tests in retina cells and in vivo tests for the proper drug development.

CONFLICTS OF INTEREST
The author declares no conflict of interest.

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REFERENCES


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