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# *In vitro* and *In silico* analysis of the Anti oxidant and Angiogenic potential of *Padina tetrastomatica*

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**Abstract :** Marine seaweeds are used in Chinese medicine since ages. In the current study the antioxidant potential and angiogenic potential of *Padina tetrastomatica* was investigated. The phytochemical screening showed that the acetone extract was rich in polyphenol content (43.3mg/g). The acetone extract also exhibited antioxidant potential which correlated to the polyphenol content. The angiogenic potential of *Padina tetrastomatica* was analysed by the *in vivo* chorio allantoic membrane assay. The application of the acetone extract stimulated the budding of more blood vessels compared to the control. These results suggested that the polyphenols of the acetone extract may possess angiogenic potential. Hence, the common polyphenols including Phloroglucinol, Phlorofucofuroeckol and Scutellarein 4 methyl ether were used for *in silico* interaction studies with VEGF receptor (VEGFR). Among these polyphenols, Phlorofucofuroeckol showed the best interaction with a total score of 5.34 followed by Scutellarein 4 methyl ether. The interaction results were compared with VEGF-VEGFR interaction. The results of the study put forth that polyphenols of *Padinatetrastomatica* have angiogenic potential and can be further analysed for efficient application in therapeutic angiogenesis.

**Key words:** *Padina tetrastomatica,* chorio allantoic membrane assay, Phloroglucinol, Phlorofucofuroeckol and Scutellarein 4 methyl ether, VEGFR.

## Introduction

Marine seaweeds are used in Chinese medicine since 300BC[1].On the basis of photosynthetic pigments, marine seaweeds are classified as brown algae Pheaophyta which possess fucoxanthin, red algae Rhodophyta which has phycoerythrin and green algae Chlorophyta with chlorophyll a &b as predominant pigments. Marine seaweeds are utilized as animal feed, fertilizer, nutraceuticals and staple human food. They serve as functional foods because they are rich in insoluble dietary fiber, essential amino acids, lipid and mineral content[2].They are utilized in pharmaceutical industries due to their anticancer[3,4], anti allergic[5], anti inflammatory[6, 7], anti bacterial[8], anti oxidant[9], anti diabetic and anti obesity[10] potentials.This has increased the industrial consumption of seaweeds from 3.8 million tons in 1990 to 19 million tons in 2010. Crude extracts of marine seaweeds were used in China and Japan forthe treatment of thyroid disorders, intestinal disorders, mineral and vitamin deficiencies and gynecological problems.

Angiogenesis, the development of new blood vessels from an existing one plays a major role in growth, wound healing, repair, menstrual cycle and implantation of embryo during pregnancy[11,12]. It is regulated by cytokines such as Platelet derived growth factor, vascular endothelial growth factor andFibroblast growth factor[13].Abnormal angiogenesis is associated with many pathological conditions. Inhibition of angiogenesis contributes to diabetic foot ulcer, peptic ulcer, bowel atresia and cardiovascular diseases. On the other hand, increased angiogenesis leads to tumor growth, metastasis, diabetic retinopathy, macular degeneration and

rheumatoid arthritis. Thus the regulation of angiogenesis becomes a prominent strategy in the management of various diseases[14].

Cardiovascular diseases are the primary cause of death worldwide causing 31% deaths annually. According to the WHO 2016 data sheet CVDs account for17.5 million deaths every year. The global expense on CVD is expected to reach 1, 044 billion USD by 2030. The major factor contributing to CVD is the decreased blood flow or poor vacularisation of the heart muscles. One therapeutic approach is the restoration of blood supply by improving neovascularisation. Therapeutic angiogenesis is a recent tool developed to deliver growth factors, cells or polymeric biomaterials to promote neovascularisation[15].

Marine seaweeds are rich in natural compounds which serve as drugs or lead compounds for the development of new drugs. They produce a wide variety of secondary metabolites such as alkaloids, terpenoids, oxypilins, phlorotannins, volatile hydrocarbons, glycosides and steroids. These compounds are reported to exhibit a wide range of biological activities such as antioxidant, antineoplastic, antimicrobial, anti viral ,cytotoxicity and inhibitory activities against various enzymes[16, 17, 18].

*Padina tetrastomatica* belongs to order Dictyotaceae, class Phaeophyceae and are found to grow on rocks and dead corals in the warm temperate and tropical coastal areas. It is the only genus reported to be calcified among the brown algae. The plants are soft, flat and leathery reaching 15 to 20cm in height. It is a good source of essential aminoacids, vitamins B6,B1,B2, minerals like Calcium and Iron. Further, Padina is rich in palmitic acid, stearic, mardaric and arachidonic acids. It is reported to exhibit good antibacterial and cytotoxic properties and can be used as anti fouling agents[19, 20].The current study is aimed to investigate the phytoconstituents of *Padina tetrastomatica,* and to determine its antioxidant potential and angiogenic efficiency both by *in silico and in vivo* methods.

## Materials and Methods

### Collection of Algae

The marine brown algae *Padina tetrastomatica* was collected from Kanyakumari coast of Tamil Nadu, India during the month of January. The fresh sea weed was washed with tap water to remove the epiphytes, salt and sand attached to it. The sea weed was shade dried for a week, powdered and used for further analysis.

### Extraction and Isolation

The dried seaweed powder (100g) was extracted in different solvents(300 ml) including ethyl acetate, methanol, acetone and water in an orbital shaker at room temperature overnight. The extracts obtained were filtered and the filtrate was collected. The solvents were evaporated to get a concentrated extract[21].

### Phytochemical Analysis

The extracts were analysed for the presence of phytochemical constituents using standard procedure[22] .The extracts were separated by thin layer chromatography using thin layer chromatography strips.

### Estimation of total polyphenol content (TPC)

The total phenol content was quantified using the Folin–Ciocalteau method[23]. 100 µl of each extract was mixed with 2 ml of $Na_2CO_3$ (2%) and incubated for 2 min at room temperature. 100 µl of Folin–Ciocalteau phenol reagent was added and incubated for 30 minutes in dark at room temperature .The absorbance was measured at 720 nm. The total phenol content of the extracts was expressed as mg gallic acid per gram.

### Assay of free radical scavenging activity (DPPH activity)

The free radical scavenging activity of the algal extract was assessed by the DPPH assay[24]. Different concentrations of the seaweed acetone extract (0.1-2000µg) were added with equal volume of DPPH (60µM) solution. The reaction mixture was mixed thoroughly and incubated in dark at room temperature for 30 minutes. Ascorbic acid was used as positive control. The absorbance was read at 517nm.

***In silico* analysis of algal compounds**

The structures of polyphenols namely phloroglucinol, phlorofucofuroeckol and scutellarein 4 methyl ether widely distributed in brown algae were retrieved from Pubchem data bank (www.ncbi.nlm.nih.gov/pubchem) and interacted with X ray crystal structure of VEGF receptor (*Homosapiens*) (PDB ID:5fv1) downloaded from protein databank (http//www.rscb.org/pdb).The interaction was analyzed in SYBL X1.3 software package (http://www.tripos.com). The best interaction was determined on thebasis of total score (T score) and consensus score (C score) generated. For each interaction three best poses were recorded and the interacting residues and bond lengths were determined. The interaction was viewed in pymol a python based tool for visualization.

***In vivo* CAM assay for identification of angiogenesis**

The CAM assay was carried out as described earlier with slight modifications[25]. The fertilized white leghorn chicken eggs were procured from TANUVAS, Potheri, Chennai, India and incubated at 37 ºC and 60% humidity in incubator. A small window was made on the shell on day 3 to prevent the CAM layer from getting attached to the inner egg shell membrane. The window was resealed and the eggs were incubated till day 10. On day 10, the window was opened again and 30mm diameter filter paper disk impregnated with 50µl of acetone extract (100 ppm -1000ppm) was inoculated in the nerve region of the developing chick embryo.Disc containing acetone was used as control. The window was again resealed and the eggs were incubated for 48 hrs at 37 ºC and 60% humidity. On day 12, the window was opened and the filter paper discs were removed. The point of inoculation was analyzed for stimulation or inhibition of angiogenesis.

**Results and Discussion**

The phytochemical analysis of *P.tetrastomatica* revealed the presence of various secondary metabolites including alkaloids, glycosides, phytosterols, oils and flavonoids in the acetone, methanol and ethyl acetate extracts, whereas the aqueous extract showed the presence of glycosides,oils and flavonoids only (Table 1).

**Table1. Phytochemical analysis of *Padina tetrastomatica*.**

| Phytoconstituents | Acetone | Methanol | Ethyl acetate | Aqueous |
|---|---|---|---|---|
| Alkaloids | ++ | + | + | - |
| Glycosides | ++ | + | + | + |
| Saponins | - | - | - | - |
| Phytosterols | + | + | + | - |
| Fixed oils & fats | + | + | + | + |
| Resins | - | - | - | - |
| Phenol | +++ | - | - | - |
| Flavonoids | ++ | + | + | + |
| Protein | + | + | - | - |
| Coumarin | - | - | - | - |

+ Presence, +++ highly present, - absence.

The results are coinciding with previous studies [26, 27]. Polyphenols, the major source of antioxidants in brown algae were detected only in the acetone extract. The acetone extract was rich in these secondary metabolites compared to the other extracts which may be due to the nature of the solvent used for extraction. The separation of the algal extracts by thin layer chromatography (Figure 1A) also confirmed the presence of more metabolites in the acetone extract than the other extracts. The polyphenolic content was estimated as43.39 ± 3.09 mg/g in the acetone extract whereas it was not detected in other extracts. Based on the phytochemical analysis and TLC data, the acetone extract was selected for further analysis.

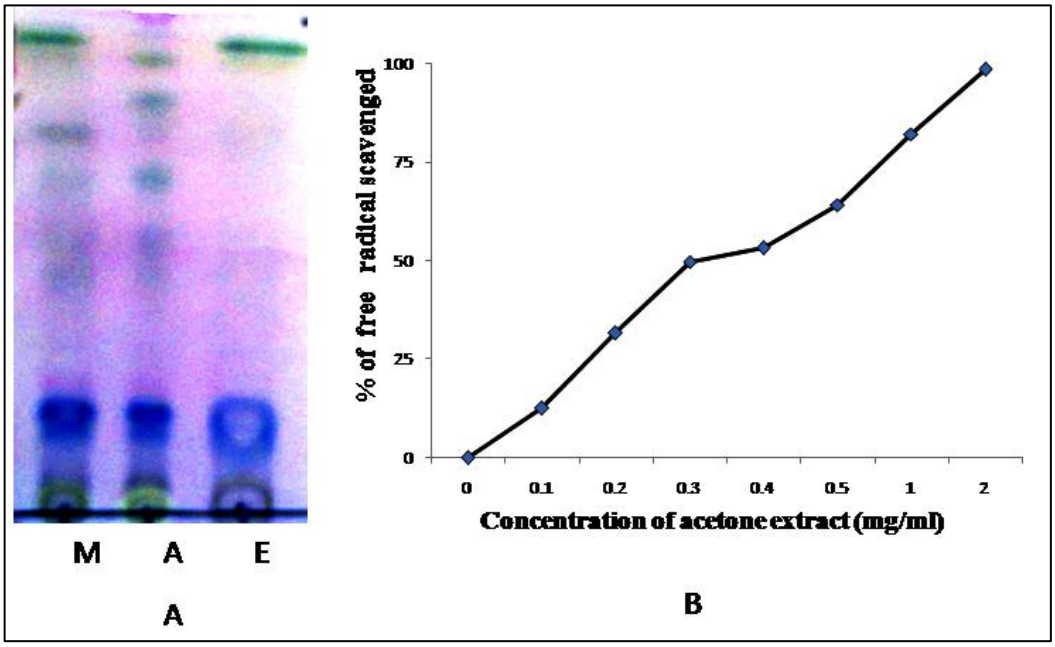


**Figure1.(A) TLC of P.tetrastomatica extracts. (B) DPPH free radical scavenging activity of acetone extract. M= methanol, A- acetone, E- ethyl acetate.**

The antioxidant activity was determined based on the percentage of DPPH free radicals scavenged. The acetone extract removed 82% of free radicals compared to ascorbic acid.The inhibitory concentration IC 50 was 300µg/ml which removed 50% of free radicals generated (Figure 1B). This anti oxidant potential may be due to the polyphenols and other secondary metabolites in the extract. The result of the antioxidant activity is directly proportional to the total poly phenol content. As polyphenols are excellent scavengers of free radicals[28], the antioxidant potential of the algae may be conferred by the polyphenols present in it. These results are similar to a previous study [29]. Previous studies have shown that Phlorotannin and bipolar phenols contribute to the antioxidant potential of brown algae[30].As phloroglucinol, phlorofucofuroeckol and scuetellarin 4methyl ether are the common polyphenols present in brown algae[31] the current study aimed to analyse the efficacy of these compounds under *insilico* in inducing angiogenesis.

The structures of these polyphenols were derived from pubchem and subjected to molecular docking with VEGF receptor. Based on the SYBL X1.3 interaction results, the algal polyphenol phlorofucofuroeckol showed the best interaction with VEGFR with a total score of 5.346 and consensus score of 5. This interaction was stabilized through residues SER 60, GLU 64,SER 52, TYR 25, CYS 26,102 and 104 in the active site of VEGFR. The interaction with scuetellarin 4 methyl ether and VEGFR yielded a T score of 4.808 and C score of 4. The interacting residues were identified as SER 52, TYR 25, CYS 104 and HIS 50. The interaction with phloroglucinol showed the least score among the polyphenols interacted yielding a T score of 3.458 and C score 4 with SER 52,TYR 25 and CYS 104 as the interacting residues(Figure 2). This was followed by the analysis of interaction between VEGF and VEGFR. The interaction was stabilized by the residues GLN 37,PRO 70,THR 31,GLU 44,LYS 10,GLU 64,LYS 107and CYS 104 of VEGFR (Figure 3). The bond length was between 1.5 to 1.8Å. The results of the interaction study thus confirmed the ability of the algal polyphenols to interact with VEGFR, the major target for binding the angiogenic cytokines. Further, the results revealed that the algal polyphenols interacted with the common residues such as SER 52, TYR 25 and CYS 104 of VEGFR. Also the residues CYS 104 and GLU 60 are the common interaction sites for both VEGF and phlorofucofuroeckol. Thus the results of the docking analysis established the efficiency of algal polyphenols to interact with VEGFR. Following this the *in vivo* determination of angiogenic activity by CAM assay was carried out.

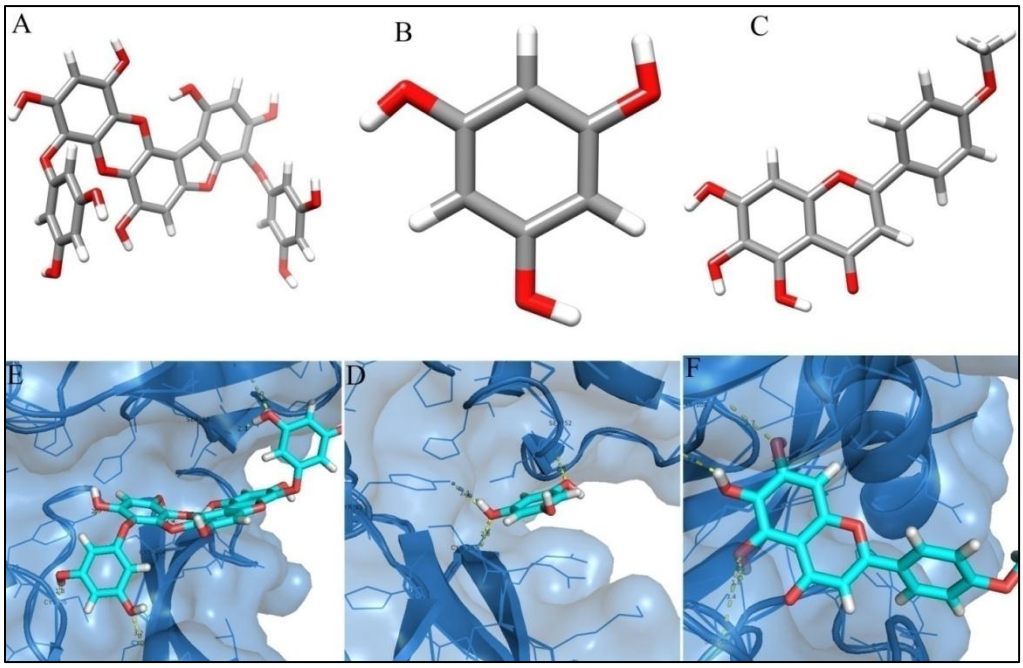


**Figure2. Interaction of polyphenols with VEGFR. (A) Structure of phlorofucofuroeckol. (B) Phloroglucinol. (C) Scuetellarin 4 methyl ether. (D) Interaction of VEGF with phlorofucofuroeckol (E) Interaction of VEGF with phloroglucinol (F) Interaction of VEGF with scuetellarin 4 methyl ether.**

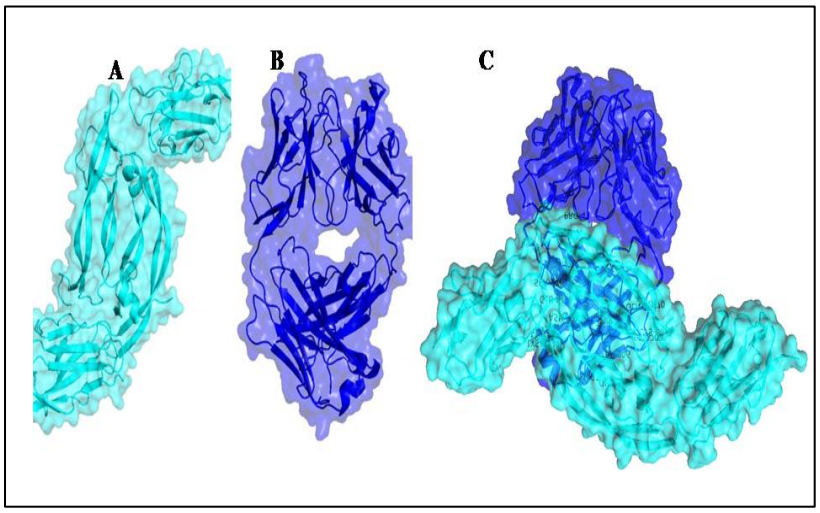


**Figure 3*In silico* analysis of VEGF interaction with VEGFR. (A) Structure of VEGFR. (B) Structure of VEGF. (C) Interaction of VEGF with VEGFR.**

The Chorio allantoic membrane (CAM) assay is widely used to analyse angiogenesis *in vivo*[32,33]. The results of the CAM assay (Figure4) showed that treatment with acetone extract induced the formation of new blood vessels compared to the control. The induction of new blood vessels after inoculation with acetone extract showed the efficiency of the extract to stimulate blood vessel sprouting.

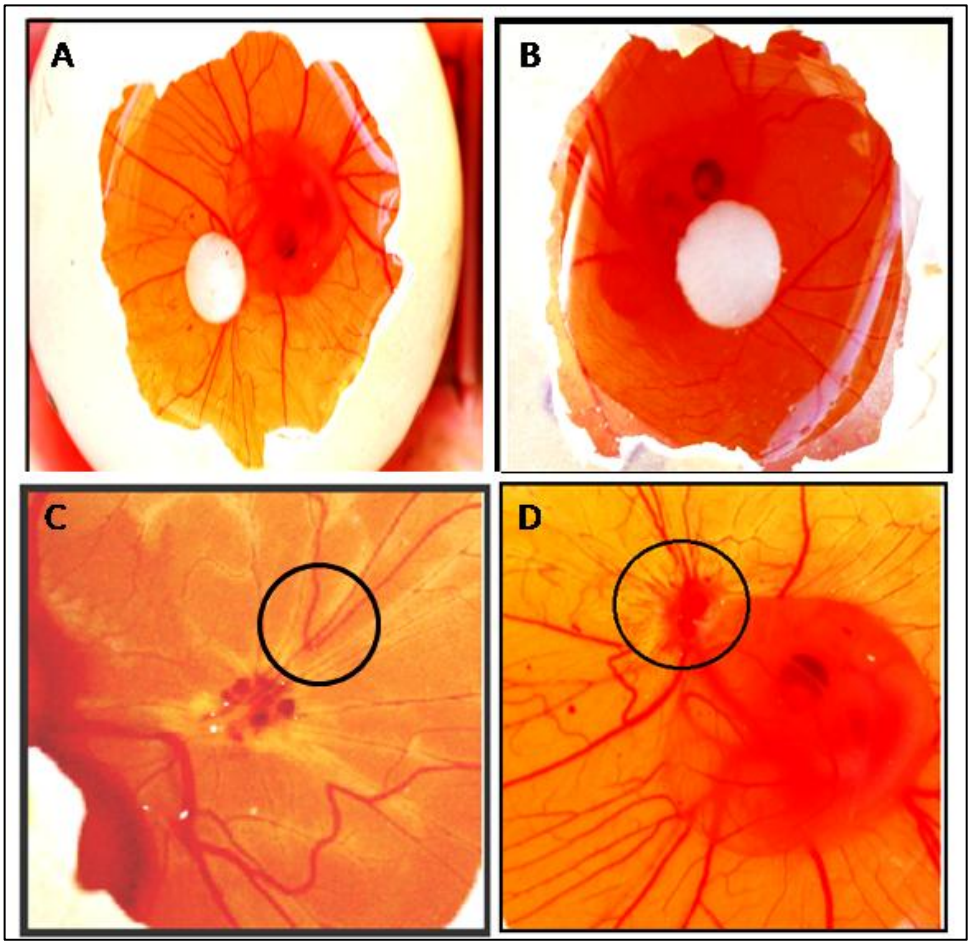


**Figure3. Effects of acetone extract on neovascularisation in chick chorioallantoic membrane. CAM vasculature on day 10 treated with (**A**) vehicle alone (**B**) acetone extract of *P.tetrastomatica*.CAM vasculature on day 12 treated with (C) vehicle (D) acetone extract. The treatment with acetone extract of Padina induced budding of blood vessels compared to control.**

The results of CAM assay suggested that the seaweed extract has proangiogenic compound that can be used in the treatment of various diseases which are characterized by poor vasculature. Similar CAM assay studies have shown that algal compounds can inhibit angiogenesisand are good sources of anti angiogenic compounds[32]. The results of the current study thus put forth that seaweeds are potent natural sources of bioactive compounds which have good antioxidant potential and can induce angiogenesis as shown by *in silico* and *in vivo* studies. Further analysis of identification and characterisation of the bioactives may enable the development of novel drugs and supplements for regulating and preventing diseases.

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