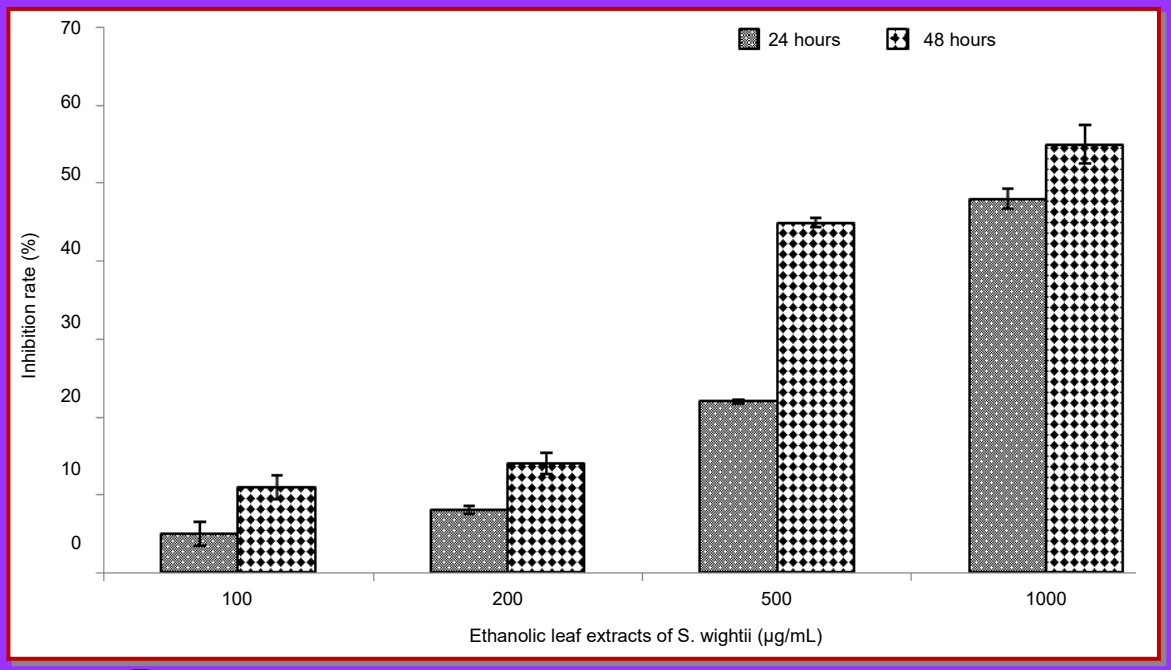

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Anti-cancer and anti-angiogenesis effect of the leaf extract of *Sargassum wightii* against osteosarcoma cancer cells

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Abstract

This study was aimed to evaluate the chemical composition of ethanolic leaf extract of a marine macro-alga *Sargassum wightii* (ELESW) followed by its vascular endothelial growth factor (VEGF) induced anti-angiogenic effect against human umbilical vein endothelial cells (HUVECs). The effect of ELESW on cell viability and its potential against *in vivo* angiogenesis was assessed by using the MTT and CAM assay respectively. The phenolic, flavonoid, polysaccharides, and protein content of ELESW were found to be 56.0 ± 4.1 mg GAE/g, 26.3 ± 3.6 mg QE/g, 284.2 ± 6.2, 100.9 ± 8.9 µg/mL, respectively. In angiogenesis, ELESW (100, 500 and 1,000 µg/mL) significantly inhibited VEGF induced angiogenesis as well as VEGF-induced proliferation and tube formation of HUVECs in a dose-dependent manner. Upon stimulition by VEGF, HUVECs rapidly increased reactive oxygen species production, which was significantly blocked by treatment with ELESW. These findings suggesting that *S. wightii* could be a valuable candidate of therapeutic efficacy.

Introduction

Most often, the osteosarcoma takes place in new developed bones of infants and adolescents accompanied by subsequent respiratory failure and lungs metastases (Marine et al., 2004; Bielack et al., 2002). Although effective curing strategies and treatments of osteosarcoma include radio surgery, and adjuvant chemotherapeutic agents including ifosfamide, cisplatin, methotrexate, doxorubicin and etoposide (Marine et al., 2004), they have frequently reported to exert adversary side effects. Moreover, these chemotherapeutic agents have displayed sever toxicity and chemoresistance in this assertive stage of cancer. This has increased a huge demand of exploring more effective and selective types of chemotherapeutic agents with less or no adverse effects to treat osteosarcoma complications (Bielack et al., 2002; Goorin et al., 1991).

Angiogenesis is a complex process of proliferation, sprouting, elongation, and migration of endothelial cells (Carmeliet et al., 2000). Since plant-derived phytochemicals are recommended as potential drug targets to treat cancer and angiogenesis-related diseases (Coussens et al., 2002).

Although significant amount of research on the biological and therapeutic efficacy of a marine alga *Sargassum wightii* has been conducted (Mizukoshi et al., 2009; Meenakshi et al., 2009), no research has been carried out so far on its efficacy to serve as a natural source of angiogenesis and osteosarcoma inhibitor with respect to its secondary metabolites. Therefore, present study was undertaken to evaluate the quantitative profile of secondary metabolites present in the ethanolic leaf extract of *S. wightii* along with its inhibitory activities against angiogenesis and osteosarcoma.

Materials and Methods

Pre-processing of sample

The seaweed *S. wightii* collected from south China coast wasproperly cleaned with tap water in order to remove all the adherent materials, packed in airtight plastic bag and immediately moved to the laboratory followed by washing with distilled water. Samples of cleaned and washed seaweed were subject to keep on blotting paper at room temperature under shadeso as to dry without losing the volatile substances from the samples. The sample was grinded to a fine powdered form for extraction purposes.

Extraction

The dried leaf powder of *S. wightii* was mixed with 70% ethanol (1:10 ratio, weight/volume) in a volumetric flask at 70°C for 1 hour followed by filtration by Whatman filter paper No. 2. The residual material was re-extracted two times with an equal volume of ethanol solvent for the same duration and temperature. Further, the filtrate sample was centrifuged (10,000 rpm) at 4°C for 15 min and supernatant was dried using a rotary evaporator and lyophilized. Lyophilized extract sample of *S. wightii* was stored at -20°C for further use.

Determination of total phenolic content

The total phenolic content in the ethanolic leaf extract of *S. wightti* was determined spectrophotometrically by Folin-Ciocalteu colorimetric method (Singleton et al., 1999). Briefly, 20 µL of diluted extract sample was mixed with 100 µL of Folin-Ciocalteu reagent and kept for 3 min at room temperature. Further, 80 µL (10% aqueous sodium carbonate solution) was added to the reaction mixture and the reaction solution was allowed to stand for 1 hour at room temperature. The absorbance of the resulting blue colored mixture was measured at 765 nm against a blank sample containing only the solvent (200 µL). The amount of total phenolics was calculated as gallic acid equivalents (GAE) from the calibration curve plotted from gallic acid standard solution and was expressed as mg GAE per g dry mass.

Determination of total flavonoid content

Total flavonoid contents of ethanolic leaf extract of *S. wightii* were determined by the colorimetric method as reported previously (Sakanaka et al., 2005). Briefly, a 100 µL of sample extract or standard compound and 400 µL of ethanol were mixed with the 500 µL (2% solution of $AlCl_3$)diluted in distilled water followed by incubation for 1 hour at room temperature and the absorbance of reaction mixture was measured at 430 nm. Quercetin was used as a reference compound to plot the standard curve, and the results were expressed as mg of quercetin equivalents (QE) per g dry mass.

Determination of polysaccharide and total protein contents

The polysaccharides and protein contents in the ethanolicleaf extract of marine alga *S. wightii* were determined using the phenol-sulfuric acid method (Masuko et al., 2005) and Bradford assay (Bradford et al., 1976) respectively.

Evaluation of anti-angiogenic activity

Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) were grown in a 0.2% gelatin-coated flask supplemented with endothelial cell growth supplement (EGM-2), consisting of fetal bovine serum (FBS), hydro-cortisone, human fibroblast growth factor-B, VEGF, long R3 insulin-like growth factor-1, ascorbic acid, human epidermal growth factor, GA-1000, and heparin. The HUVECs between passages 2 and 6 were used in the experiments.

Cell viability assay

The efficacy of ethanolic leaf extract of *S. wightii*on cell viability was assessed using the MTT staining method (Lee et al., 2008). The HUVEC cells (4 to 5-days-old cultures) with a density of 2 x 10^4 cells per well were seeded in a 96-well plate. Control cells were grown in the same medium containing sample-free vehicle. Further, cells were incubated with ethanolic leaf extracts of *S. wightii*at different concentrations (100, 500, 1000, 2000 µg/mL) for 48 hours. Then, 20 µL of MTT was added to the wells, and cells were incubated for extra 4 hours. A 200 µL of dimethyl sulphoxide (DMSO) was added to each culture and mixed properly in order to dissolve the reduced MTT crystals. Relative cell viability was determined by scanning with a microplate reader with a 540 nm filter.

Cell proliferation assay

Cell proliferation assay was performed according to the method as described previously (Lee et al., 2008). The HUVEC cells coated (density 2 x 10^4 cells per well) on gelatin-based 48-well micro titer plate were incubated for 24 hours in EBM-2, containing EGM-2 followed by washing with EBM-2 after 24 hours and incubated for 6 hours in EBM-2, containing 1% FBS. The coated HUVEC cells were then co-treated with various concentrations of ethanolic leaf extracts of *S. wightii* (100, 500 and 1000 µg/mL) and 20 ng/mL of VEGF solution. After incubation for 48 hours, the numbers of viable cells were measured using MTT assay.

Chick chorioallantoic membrane (CAM) model of angiogenesis

In this model, the anti-angiogenic efficacy of ethanolic leaf extract of *S. wightii* was evaluated following a previously reported method (Lee et al., 2008). In this assay, a hypodermic needle was used to make a small

hole in the shell of fertilized chicken eggs concealing the air sac. In addition, another hole was made on the broad side of the egg directly covering the vascular portion of the embryonic membrane. A window of about 1.0 cm^2 was then cut into the shell over the dropped CAM which allowed direct access to the underlying CAM. To induce new blood vessel branches on the CAM of 10 days old embryos, VEGF was used as a standard pro-angiogenic agent. Dried sterile discs pretreated with cortisone acetate (3 mg/mL) were suspended in 0.1 M PBS containing VEGF, while control discs were suspended in 0.1 M PBS without VEGF, and placed on growing CAMs for 30 min. Further, ethanolic leaf extract of *S. wightii* at different concentrations (100, 500 and 1000 µg/mL) was added topically to the CAMs on previously placed discs and the samples of treated CAMs were incubated for 3 days at 37°C and 55% relative humidity. The numbers of vessel branch points contained in a circular region of treatment and control tissues were counted (image data not shown). The resulting angiogenesis index was measured as the mean ± SD of the new branch points for each set of samples.

Effect of *S. wightii* on osteosarcoma

Cell lines and cell culture

The human epithelial OS cell line, U2-OS, was cultured in Dulbecco's modified Eagle's medium and supplemented with fetal bovine serum (10%) in a humidified and 5% CO_2 incubator at 37°C. All chemicals and reagents were purchased from Sigma-Aldrich, MO, USA.

Osteosarcoma cell growth assay

This assay was performed using a previously reported method (Wang et al., 2013). In brief, the U2-OS cell line was cultured in a 96-well tissue culture plate with cell density of 5 × 10^3 cells per well, in Dulbecco's modified Eagle's medium containing 10% FBS and 2 mM L-glutamine. Upon adherence, the medium was replaced after 12 hours, and the cells were incubated with different concentrations (100, 500 and 1000 µg/mL) of ethanolic leaf extracts of *S. wightii* followed by subsequent analysis by MTT assay. The assay was performed in three replicates at 490 nm.

Osteosarcomacell viability assay

The inhibitory effect of the ethanolic leaf extract of *S. wightii* on the viability of osteosarcoma cells was performed as reported previously (Peng et al., 2013). In brief, SaOS-2 cells were seed cultured at the density of 3 × 10^3 cells per well in a 96-well culture plate for overnight. Then the medium was replaced with fresh medium, and the inhibitory effect of different concentrations of ethanolic leaf extract of *S. wightii* (100, 500 and 1000 µg/mL) was measured after 24 hours. The determination of cell viability was carried out by CCK-8 assay. In brief, the CCK-8 solution was mixed with SaOS-s cells in a 96-well plate, followed by incubating them at 37°C for 60 min. Absorbance was read at 570 nm.

Results

In this study, the amount of total phenolic content in the ethanolic leaf extract of *S. wightii* was expressed as GAE/g dry mass obtained from the standard calibration curve of gallic acid as reference drug. The content of total phenolics in ethanolic leaf extracts of *S. wightii* was found to be 56.0 ± 4.1 mg GAE/g (Table I). In this study, the content of flavonoid compounds in

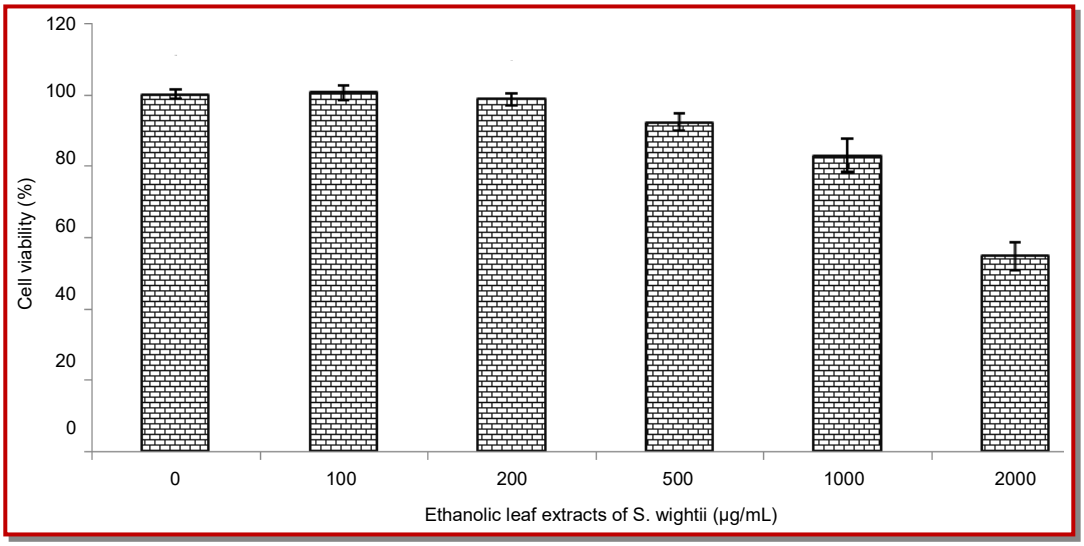

Figure 1: Effects of ethanolic leaf extract of *S. wightii* on cell viability. The number of viable cells was measured by using MTT assay. Results are mean ± SD; n = 3; Reduction observed at 2,000 µg/mL

Table I

Chemical composition profile of ethanolic leaf extract of *S. wightii*

Chemical components	Ethanol extract
Total phenolic content (mg GAE/g)	56.0 ± 4.1
Total flavonoid content (mg QE/g)	26.3 ± 3.6
Polysaccharide (µg/mL)	89.7 ± 3.8
Protein (µg/mL)	100.9 ± 8.9

Results are mean ± SD; n = 3; [a]p<0.001

ethanolic leaf extract of *S. wightii* was expressed as QE/g dry mass, and was found to be 26.3 ± 3.6 mg QE/g. Moreover, the ethanolic leaf extract of *S. wightii* revealed 284.2 ± 6.1 µg/mL and 100.9 ± 8.9 µg/mL of polysaccharide and total protein content, respectively.

The cytotoxic effect of ethanolic leaf extracts of *S. wightii* against HUVEC cells was determined by MTT assay so as to define the effective concentration needed for treatment. Exposure of HUVECs to ethanolic leaf extract of *S. wightii* for 48 hours did not reduce cell viability. However, a reduced viability pattern in the HUVEC cells was observed to the exposure of cells at 2000 µg/mL of ethanolic leaf extract of *S. wightii* for 48 hours as compared to control (Figure 1). The results obtained in this assay confirmed that ethanolic leaf extract of *S. wightii* did not affect the viability of HUVEC cells at concentrations lower than 2000 µg/mL.

Further, in this study, the inhibitory effect of ethanolic leaf extract of *S. wightii* as its anti-angiogenic efficacy was determined on VEGF-induced cell proliferation assay in HUVEC cells. HUVEC cells treated at various concentrations (100, 200, 500, and 1000 µg/mL) ethanolic leaf extract of *S. wightii* resulted in the significant inhibition of VEGF-induced HUVEC proliferation by 120.2 ± 0.5, 110.9 ± 3.3, 101.1 ± 1.3, 91.0 ± 4.1%, respectively. The results on proliferation rate on HUVEC cells were found in a dose-dependent manner (Figure 2).

Moreover, the ability of ethanolic leaf extract of a marine alga *S. wightii* to inhibit *in vivo* angiogenesis was determined by CAM assay. The results obtained in this assay showed that numbers of blood vessel branch points significantly increased upon VEGF treatment as compared to control treated with only PBS. However, exposure to ethanolic leaf extract of *S. wightii* significantly suppressed VEGF-induced angiogenesis which was in a concentration-dependent manner (Figure 3).

The MTT assay revealed the inhibitory effect of the ethanolic leaf extract of *S. wightii* (100, 200, 500, and 1,000 µg/mL) against the growth of U2-OS cell line. Based on the growth curve observed, it was found that the U2-OS cells were found sensitive to ethanolic leaf extract of *S. wightii* and the inhibitory effect was in a concentration dependent manner (Figure 4A). These findings indicated that the ethanolic leaf extract of *S. wightii* was able to inhibit U2-OS cell proliferation *in vitro*.

In addition to this, cytotoxic effect of the ethanolic leaf extract of *S. wightii* (100, 200, 500 and 1,000 µg/mL) was determined against U-2 OS cells for 48 hours through flow cytometry and the percentage of viable cell counts was measured. In this study, higher concentration of *S.*

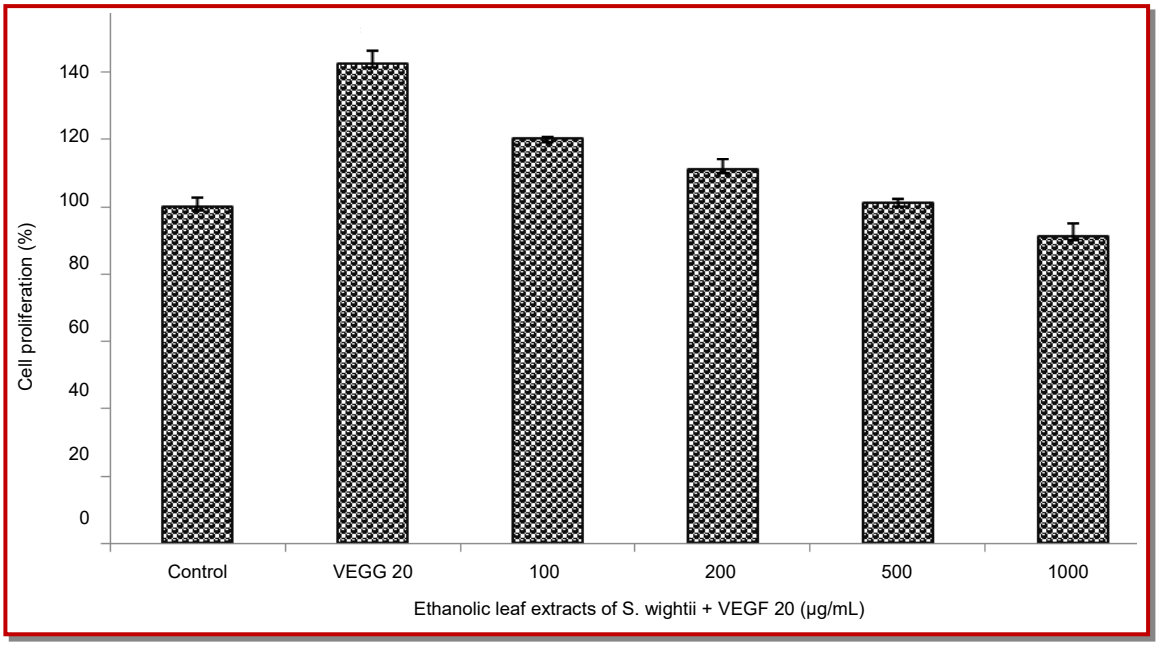

Figure 2: Inhibitory effect of ethanolic leaf extract of *S. wightii* on VEGF-induced proliferation. The number of viable cells was measured by using MTT assay. Results are mean ± SD; n = 3

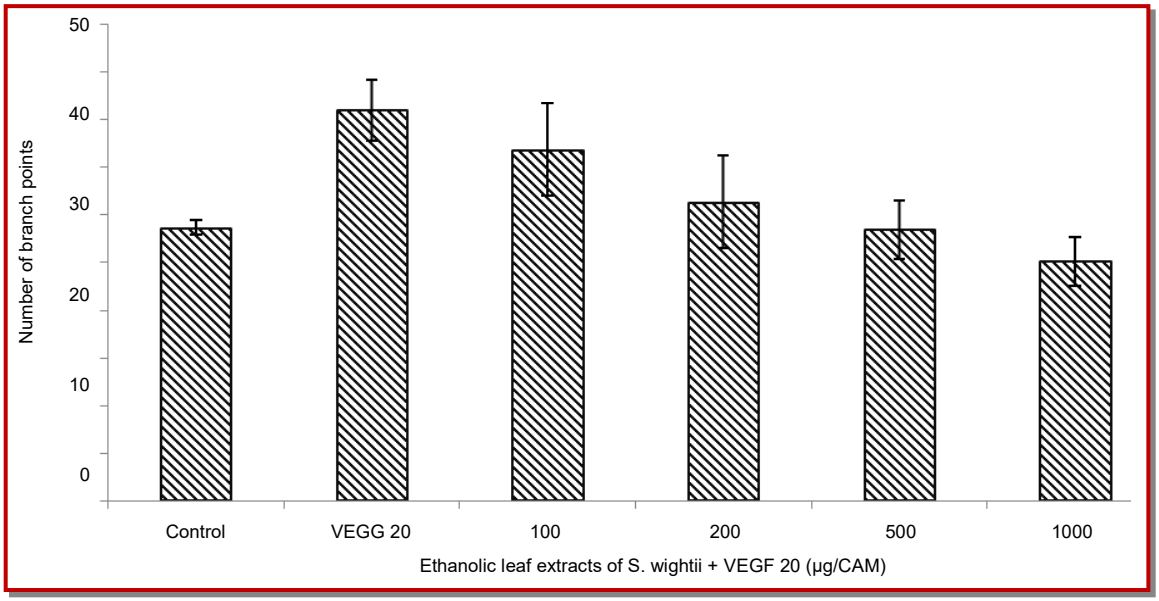

Figure 3: Inhibitory effects of ethanolic leaf extract of *S. wightii* on VEGF-induced angiogenesis. Results are mean ± SD; n = 3

wightii showed significant reduction on the cell viability (Figure 4B). Moreover, this study also revealed the effect of various concentrations of the ethanolic leaf extract of *S. wightii* on U2-OS cells and tumor cell proliferation and apoptosis were measured. The findings confirmed that the ethanolic leaf extract of *S. wightii* significantly inhibited the cell proliferation. The effect was time and dose-dependent with increased rate of apoptosis.

Discussion

The maximum protein content was recorded in the brown alga *S. wightii* by (Seenivasan et al., 2012) which is supportive to the present study that the marine alga *S. wightii* showed highest protein content. Moreover, phenolic compounds are considered to be most significant and biologically active secondary metabolites with various health beneficial properties (Vdnere et al., 2012). In addition, flavonoids are ubiquitously distributed in plants and human diet with most common group of polyphenolic compounds. Among the so far discovered flavonoids, quercetin is known to be the best plant-based flavonoid with multitude of biological and functional properties (Davis et al., 2009). In the present study the high content of total phenol and flavanoid was observed.

Several food-derived compounds display antiangiogenic activity through single or multiple step angiogenesis signaling path way such as capsaicinmediated inhibition of VEGF-induced tube formation in HUVECs and by activating VEGF-induced molecular signaling pathway (Gupta et al., 2010). Belloni et al., 2007 reported that VEGF is the most potent and well recognized angiogenic cytokine and angiogenic inducer of proliferation, migration, and blood vessel formation in HUVEC cells. These reports are in line with the present research findings. Similar results have been reported previously where allinin from garlic displayed significant inhibitory effect on fibroblast growth factor-2 (FGF-2)-induced tube formation and CAM angiogenesis (Mousa et al., 2005). Hence, it may be said that the anti-angiogenic activity of *S. wightii* might be correspond to the phytochemicals such as phenolics and flavonoids present in large amount in the ethanolic leaf extract of *S. wightii*.

Angiogenesis is considered an efficient target in tumor therapy since it is crucial for the growth of solid tumors not only by supplying oxygen and nutrients for the survival of tumor cells but also provides a route for metastatic to spread (Cooney et al., 2006). It has been confirmed that expression of VEGF is closely linked with angiogenesis and its inhibition is considered a well defined parameter in anti-cancer strategy (Kunnumakkara et al., 2007). In this study, it was observed that ethanolic leaf extract of marine alga *S. wightii* effectively inhibited HUVEC cell tube formation. Recent study reported by Peng et al., 2013 confirmed that a phytochemical, thymoquinone from *Nigella sativa* can inhibit tumor angiogenesis and was able to prevent osteosarcoma cell growth in a mouse osteosarcoma model.

Osteosarcoma is considered to be the most common bone tumor at primitive stage of infants. Moreover, development of drug-resistance in current chemotherapeutic, and disease metastasis has also become the major hurdles in anti-cancer therapy. This has led scientists to find new and innovative chemotherapeutic

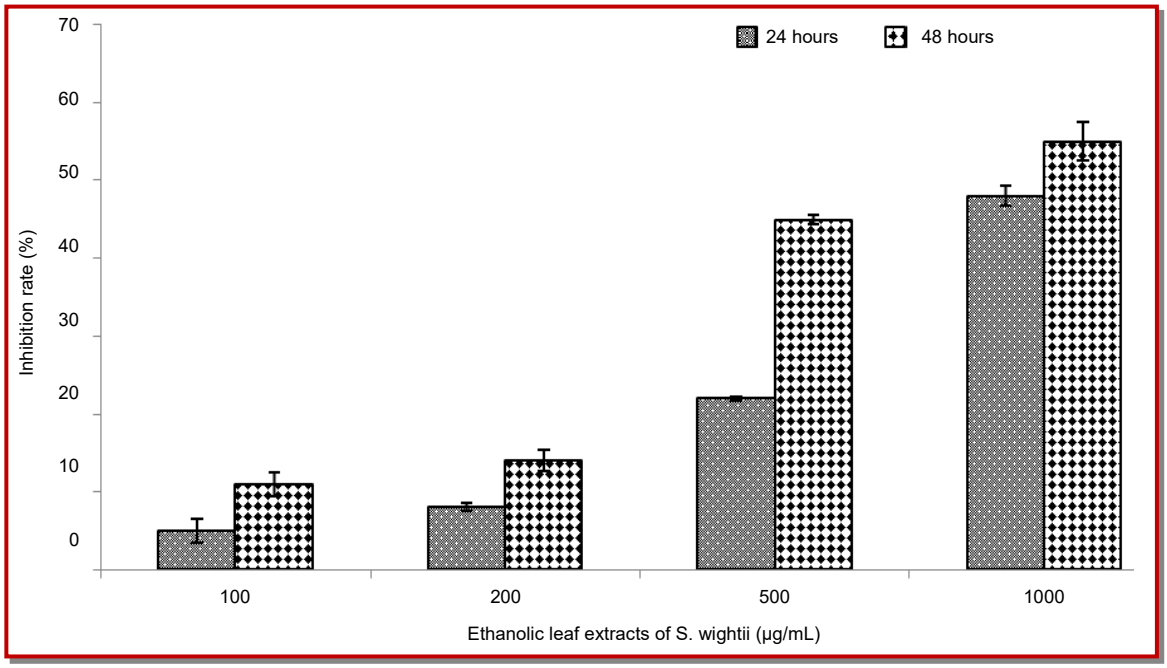

Figure 4A: Effect of ethanolic leaf extract of *S. wightii* on suppresses U2-OS cell proliferation. Results are mean ± SD; n = 3

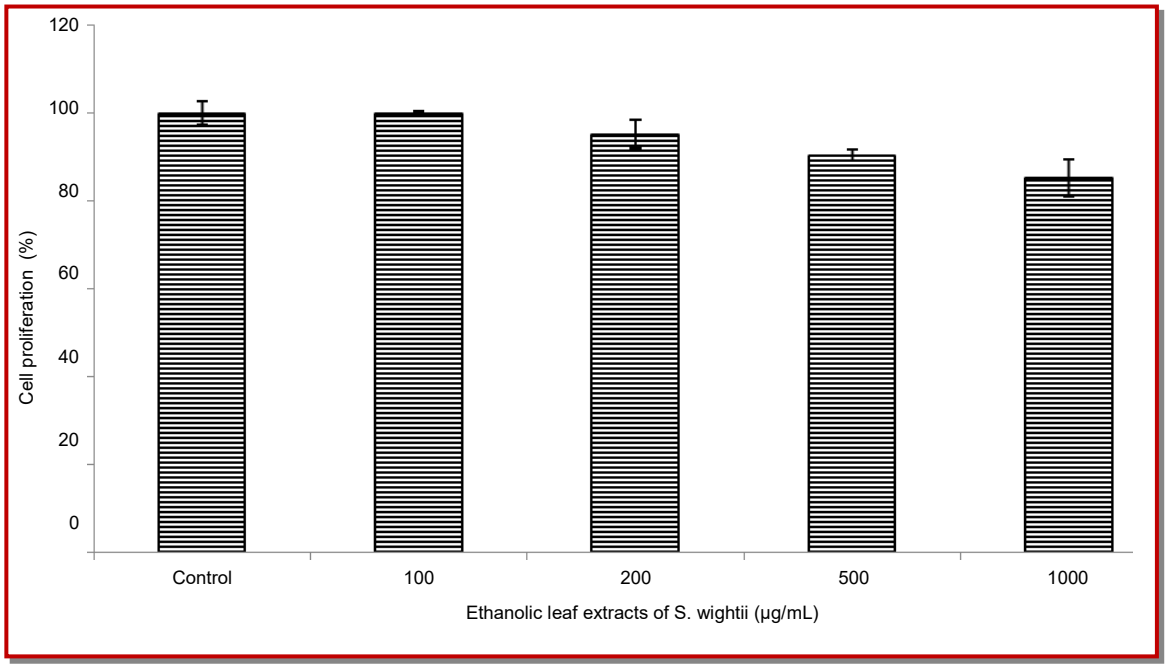

Figure 4B: Inhibitory effect of the ethanolic leaf extract of *S. wightii* on the growth of the U2-OS cells cell line. Results are mean ± SD; n = 3

agents of plant origin which are less toxic and environmental friendly. A large number of marine resources have been screened for the development of new bioactive compounds to combat against dreadful diseases. However, seaweeds or marine algae have provided huge variety of therapeutic agents with biomedicinal potential especially various species of *Sargassum* which have shown profound biological activities including antiviral, anti-cancer and antitumor efficacy (Kaliaperumal et al., 2003). Our study indicated that the ethanolic leaf extract of *S. wightii* have potential to inhibit tumor cell proliferation and induces apoptosis in U2-OS cells.

Conclusion

The ethanolic leaf extract of *S. wightii* possesses preventative therapies against angiogenesis-related diseases.

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Conflict of Interest

Authors declare no conflict of interest

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