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Extract of Indian Green Mussel, *Perna viridis* (L.) Shows Inhibition of Blood Capillary Formation *in vitro*

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ABSTRACT

The extract of the Indian green mussel (*Perna viridis* L.) was found to inhibit the formation of endothelial cell capillary tube in a concentration dependent manner *in vitro*. At a concentration of 5 mg/ml of crude extract, there no formation of intercellular junctions and capillary tubes was observed, even after 6 hours. However, at the concentrations below 1 mg/ml, a few cellular junctions and formation of capillaries began to increase at both concentrations of 0.2 and 0.04 mg/ml. The aqueous fraction was also found to inhibit the capillary and junction formations at 5 mg/ml, but it was less effective at lower concentrations, as compared to the total crude extract. The methanol (MeOH) extract was more active than that of the aqueous extract; hence, it was further fractionated into 5 fractions (F1 to F5) and tested for the presence of angiogenic activity. Fraction 3 (F3), which was used at 100 µg/ml concentration, showed a significant inhibitory effect on the formation of intercellular junctions and tube, whereas the other fractions did not show any activity.

Keywords: Indian green mussel, *Perna viridis* (L.), anti angiogenic activity

INTRODUCTION

Substances which promote or inhibit angiogenesis are of clinical importance. Most of the known derivatives have so far reported short half-lives and undesirable side effects, and thus considerably limiting considerably their use in clinical practices. Therefore, it is important to search for and discover novel molecules in controlling angiogenesis without major side effects. A number of compounds from marine organisms possessing anti-bacterial, anti-coagulant, anti-diabetic, anti-inflammatory, anti-fungal, anti-malarial, anti-tuberculosis and anti-viral properties have recently been reported (Mayer and Hamann, 2005). Similarly, some compounds have been found to significantly affect the cardiovascular and nervous systems (Mayer and Hamann, 2004).

The preliminary attempt to identify the presence of angiogenic modulators, in the crude extract from the Indian green mussel *Perna viridis* (L.), revealed that some of the fractions of the crude extract promoted vasculogenesis in chick, while others showed an inhibitory effect (Bichurina *et al.*, 1994). This finding indicated that the crude extract was likely to contain both substances which promote and inhibit vasculo-endothelial development. This interesting observation prompted the researchers to envisage further investigation on the possible role of the active substances contained in these extracts on the formation of blood capillary and angiogenesis at large.

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MATERIALS AND METHODS

Cells Cultures

Human microvascular endothelial cells (HMEC) (Golestaneh *et al.*, 2001), were cultured in endothelial basal medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin (PAA Laboratories Inc, Etobicoke, ON, USA), according to the recommendations of the supplier. To ensure endothelial phenotype of the cell lines, expression of some typical markers (vWR, E-selectin, VEGF-R2) was assessed using the PCR and immunocytochemistry.

The Preparation of Extract

The crude extract (GM) from the Indian green mussel (*Perna viridis* L.) was prepared by the process of acid enzyme hydrolysing as described by Chatterji *et al.* (2004). The extract was freeze-dried with the help of a tabletop freeze dryer (Edward Micromodulo, Germany) and stored till further use. The crude extract was sub-fractionated into methanol (MeOH) and aqueous fractions (GM Aqs). When required, all these fractions (crude, aqueous and methanol) were suspended in RPMI-1640 to achieve different final concentrations (5, 1, 0.2 and 0.04 mg/ml). In the present study, the evaluation of the anti-angiogenic activity, in these fractions, was done using the matrigel bi-dimensional models.

Bi-dimensional Angiogenesis Assay

In the bi-dimensional model (matrigel which is a standardized cancer cell extra-cellular matrix), the endothelial cells were seeded on the matrigel, in presence and absence of; a) crude (GM); b) aqueous (GM Aqs); and c) methanol (MeOH) fractions of the mussel extracts. The experiment was conducted using the growth factors-enriched matrigel to find out about the inhibitory effect on angiogenesis. The human microvascular endothelial cells (HMEC) line was continuously propagated in 75 cm culture dishes, containing RPMI 1640 supplemented with glutamine (1 mM), fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 U/ml) and incubated at 37°C, in humidified 5% CO_2 atmosphere.

To assess the formation of tubule, the HMEC were pre-incubated for 18 hrs, and the concentrations of the different fractions to be tested were increased. After the incubation period, the cells were detached by adding 200 µl of accutase, and the culture flask was again kept at 37°C for 10 minutes. 24 well plates were then coated with 100 µl of Matrigel for 2 hrs at 37°C and layered with HMEC ($2x10^5$/well), which was previously incubated for 18 hrs, with the fractions in serum-free RPMI 1640 to be tested. The control test was performed by layering the untreated endothelial cells.

Fractionation of Extract

In this study, the effects of both methanol (MeOH) and aqueous (Aqs) extracts on angiogenesis were compared. As the MeOh extract was found slightly more effective than aqueous extract, it was further fractionated by gel chromatography using Sephadex (G10), and this was followed by the reverse phase HPLC using a C-18 column [mobile phase -water (40%): Acetonitrile (60%)]. This process was found to yield five fractions. Each fraction was then freeze-dried and re-suspended (100 µg/ml) in RPMI-1640 medium with 10% FCS. After that, the five fractions were further tested to determine the most angiogenesis effective fraction using the bi-dimensional model, as described in the earlier section.

RESULTS

Crude Extract of Indian Green Mussel Inhibited the Formation of Endothelial Cell Capillary Tube

In the bi-dimension matrigel assay, the results showed that the crude extract of the Indian green mussel inhibited the formation of the endothelial cell capillary tube. Initially, the endothelial cells (when plated on matrigel) began to organize in several lines with intercellular junctions. At a later stage, the formation of capillary tubes was detected. A dose dependent inhibition of the formation of capillary tube and intercellular junctions were induced by the crude extract of the Indian green mussel (*Figs. 1, 2* and *3*). At a concentration of 5 mg/ml of the crude extract (GM), there was neither formation of capillaries nor intercellular junctions, even after 6 hours of incubation. At the concentration of 1 mg/ml, a few cellular junctions occurred, but no formation of any capillary tube was detected (*Figs. 1, 2* and *3*). Nevertheless, at both concentrations of 0.2 and 0.04 mg/ml of the crude extract, the number of intercellular junctions, as well as the formation of the capillary tube, began to increase.

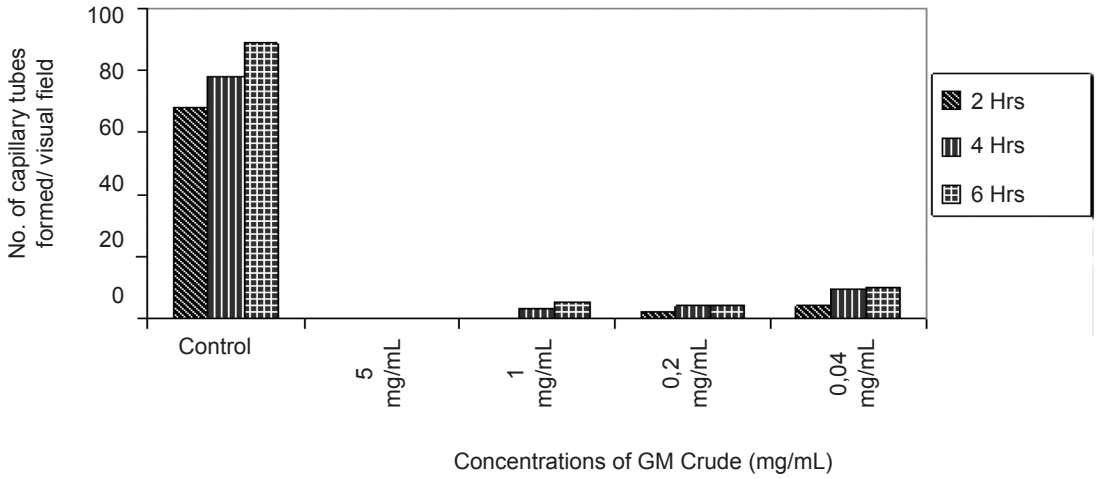

Fig. 1: Dose and time dependant effects of the GM crude on the formation of capillary tubes of human microvascular endothelial cells

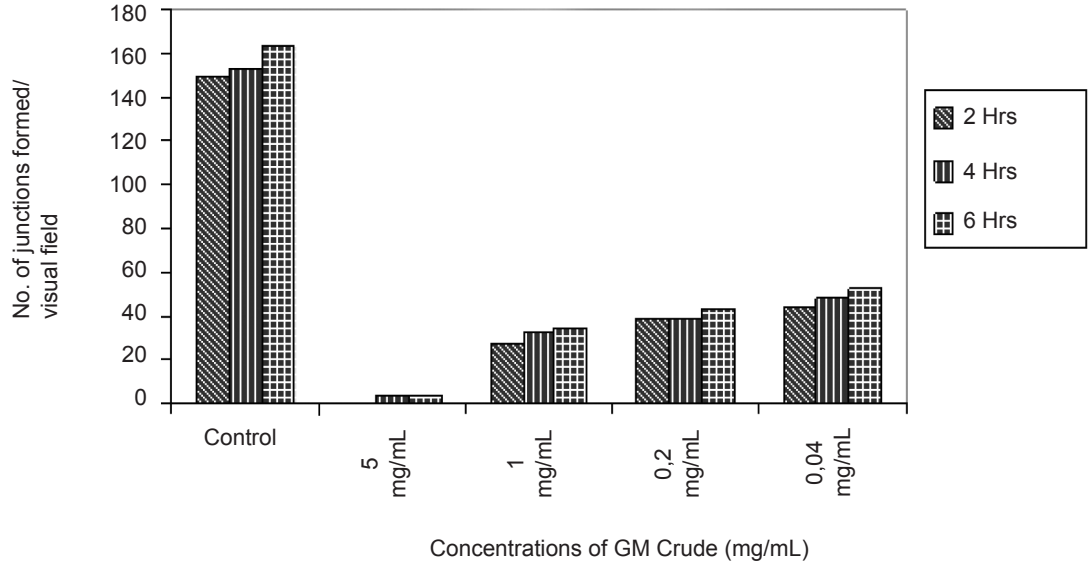

Fig. 2: The dose and time dependant effects of the GM crude on the formation of intercellular junction of human microvascular endothelial cells

Aqueous and Methanol Extracts of Green Mussel Inhibited the Formation of Endothelial Cell Capillary Tube

Although aqueous fractions (GM Aqs) was found to inhibit the formation of capillary and junction at the concentration of 5 mg/ml, it was also indicated as less effective at lower concentrations as compared to the total crude extract (*Figs. 4* and *5*).

The MeOH extract was also shown to inhibit the formation of the capillary tube and junction. At 5 mg/ml of the MeOH extract, neither cell junction nor capillary tube formation occurred (*Figs 6* and *7*). At the concentrations of 1 and 0.2 mg/ml, the MeOH extract was shown to induce a significant decrease in the number of cell junctions and the capillary formation as compared to the control.

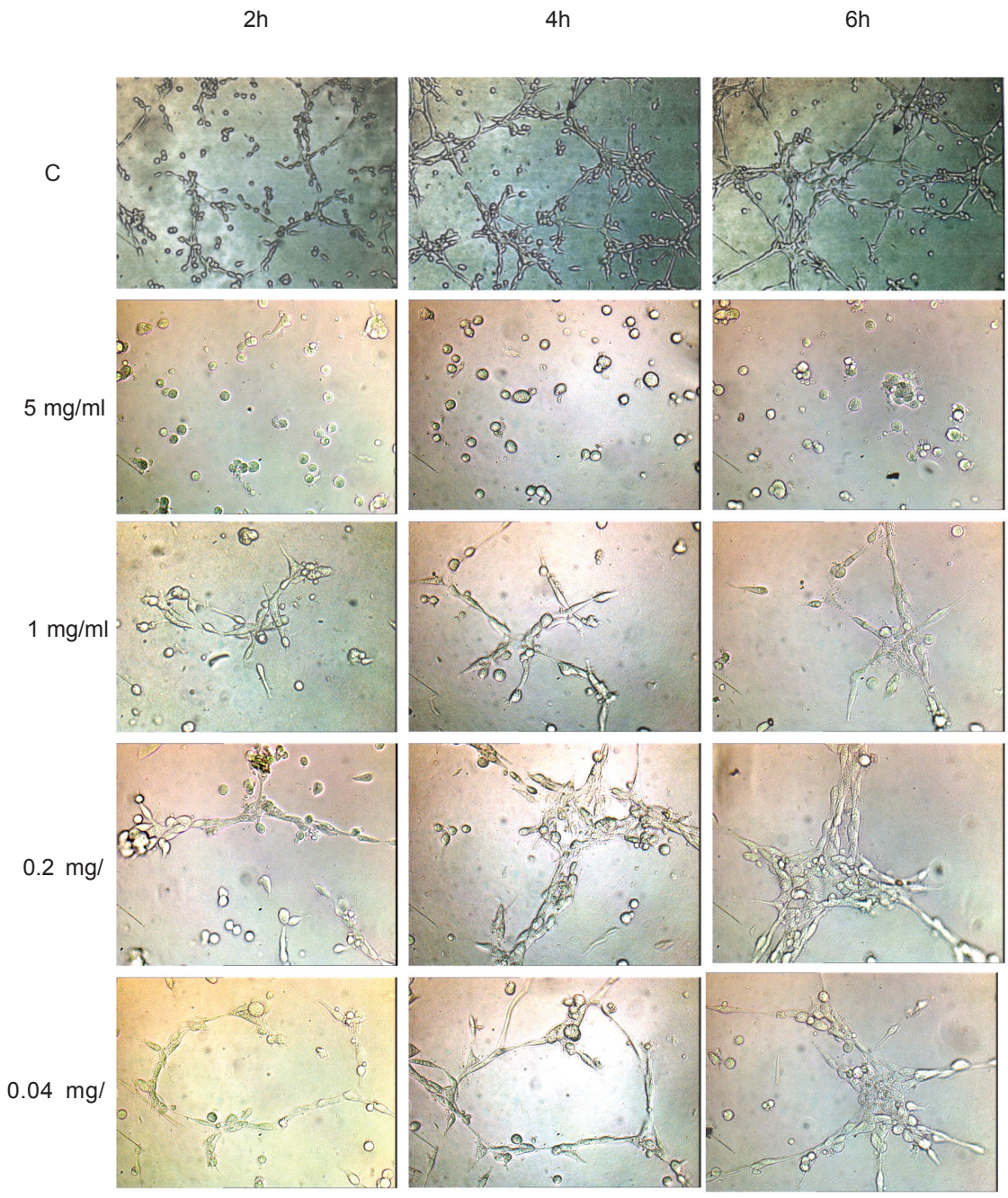

Fig. 3: A dose and time dependent inhibition of the capillary tube formation and intercellular junctions induced by the crude extract of the Indian green mussel

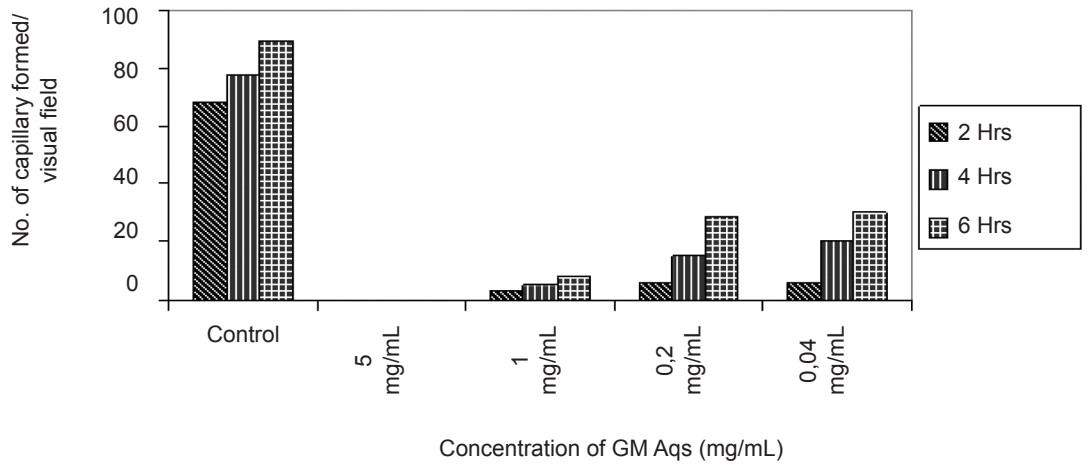

Fig. 4: Dose and time dependant effects of the GM Aqs on the formation of the capillary tubes of human microvascular endothelial cells

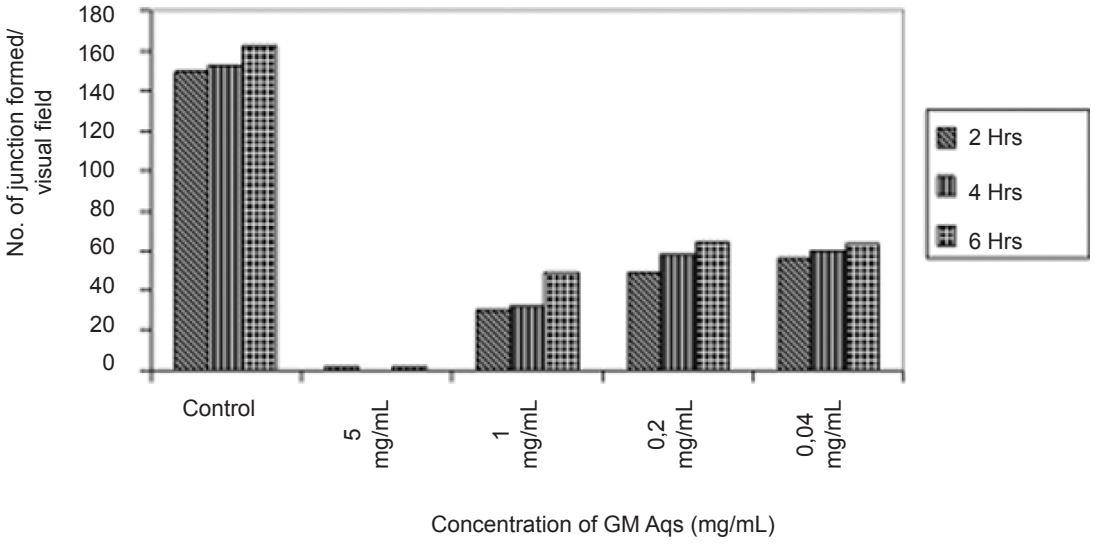

Fig. 5: Dose and time dependant effects of the GM Aqs on the formation of intercellular junction of human microvascular endothelial cells

The results presented in *Figs. 6* and *7* showed that the MeOH extract was found to be more active than that of the aqueous extract. Therefore, only the MeOH extract was used in the subsequent studies. The MeOH extract was further fractionated into 5 fractions (F1 to F5) and tested for the presence of anti-angiogenic activity even up to 18 hrs. As illustrated in *Figs. 8* and *9*, fraction 3 (F3) which was used at 100 µg/ml of the final concentration showed a significant inhibitory effect on the intercellular junctions with respect to the length of capillary (*Fig. 8*) and the number of tube formation (*Fig. 9*), whereas the other fractions did not show any notable activity.

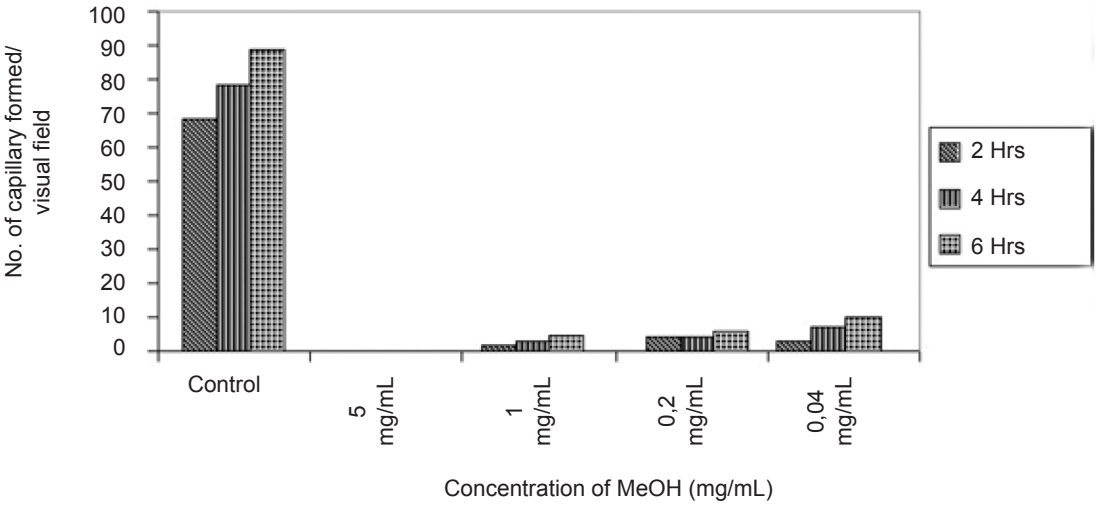

Fig. 6: Dose and time dependant effects of the GM Aqs on the formation of capillary tubes of human microvascular endothelial cells

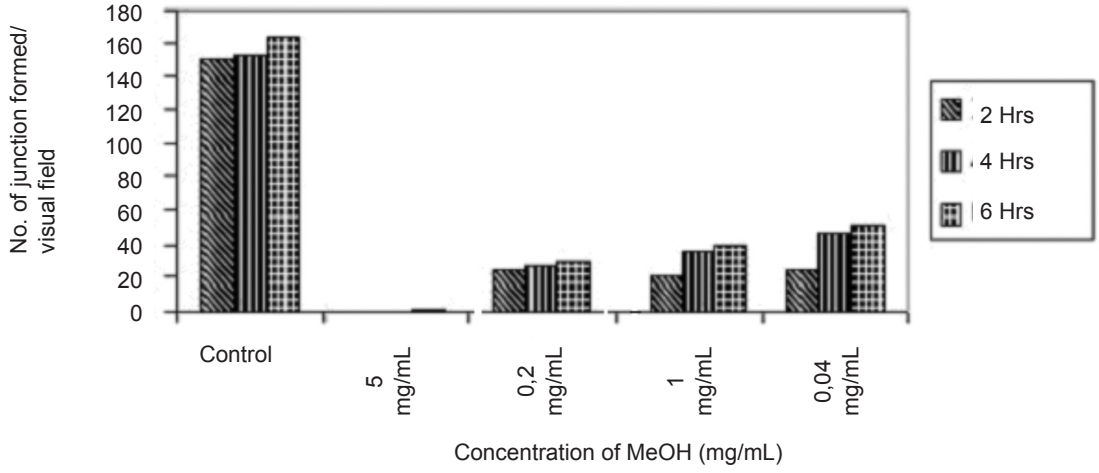

Fig. 7: Dose and time dependant effects of Meoh on the formation of intercellular junction of human microvascular endothelial cells

DISCUSSION

In the course of a screening programme to identify new pharmacologically active compounds from marine organisms, the earlier effort was made by the researchers to search for novel bioactive compounds from the Indian green mussel (Bichurina *et al.*, 1994; Chatterji *et al.*, 2002). Extracts, prepared from the Indian green mussel i.e. *Perna viridis* (L.), showed an inhibition on the formation of osteoclast and based on this finding, it could therefore be used to control osteoporosis (Rao *et al.*, 2003). It also showed the inhibition of HIV virus replication (Mitra and Chatterji, 2004) and the inhibition of replication of *Plasmodium falsiparum* (Malhotra *et al.*, 2003).

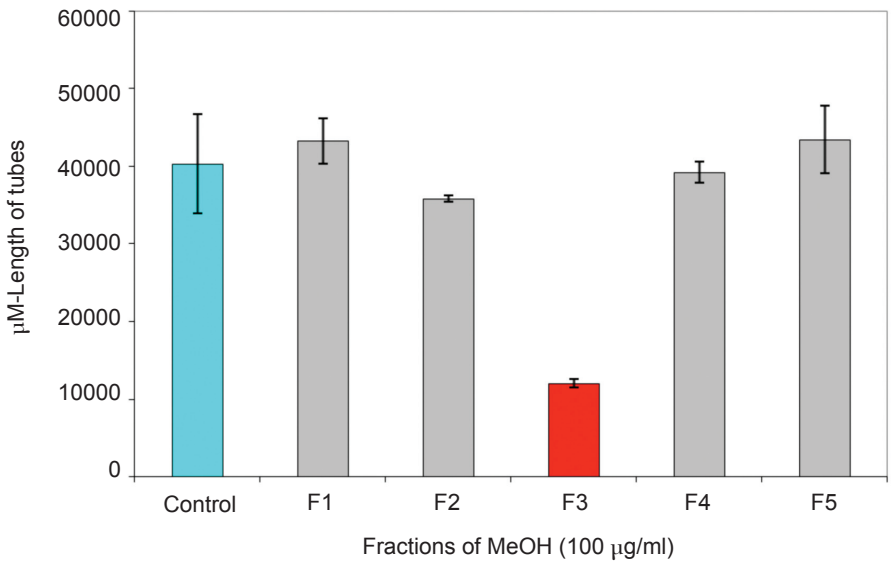

Fig. 8: The MeOH extract was further fractionated into 5 fractions (F1 to F5) and tested for the presence of anti-angiogenic activity, even up to 18 hrs. Fraction No. 3 (F3), used at 100 μg/ml of the final concentration, showed a significant inhibitory effect on the length of capillary

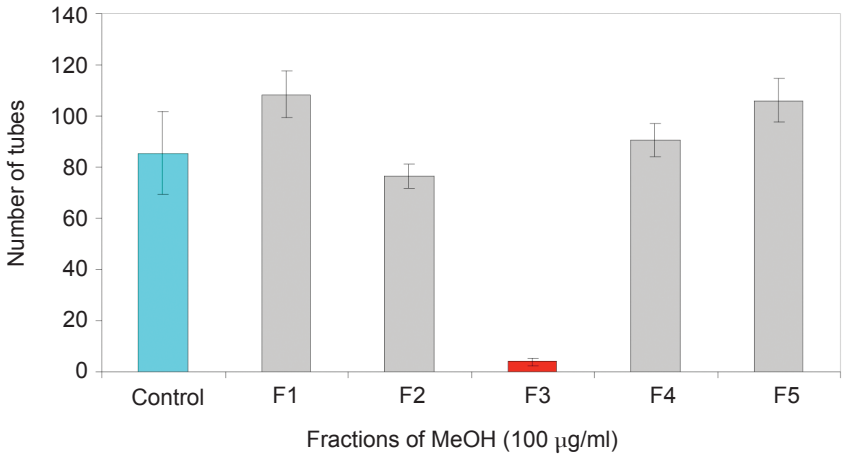

Fig. 9: The MeOH extract was further fractionated into 5 fractions (F1 to F5) and tested for the presence of anti-angiogenic activity, even up to 18 hrs. Fraction No. 3 (F3), used at 100 μg/ml of final concentration, showed a significant inhibitory effect on the intercellular junctions of capillary

Substances or molecules, having anti-angiogenic properties, are useful in cancer therapy. Anti-angiogenic substances can be used to inhibit the growth of tumour mass by preventing neo-vascularization of an early developing tumour. Therefore, such substances are of importance, not only to prevent the development of tumour, but also to get rid of the subsequent metastasis. Thus, pharmaceutical firms, which are aiming at blocking cancer cell proliferation and dissemination, have developed a large number of anti-angiogenic drugs. However, these drugs have adverse effects which include short bench life, high toxicity and high cost.

In this study, it was the first time that the identification, purification and characterization of the compound(s) from the Indian green mussel, *Perna viridis* inhibiting angiogenesis were reported. The anti-angiogenic activity of the green mussel extracts, characterized by a decrease in capillary tube formation, should have interesting applications in several pathologic events such as cancer, inflammation, and age-related macular degeneration (AMD), i.e. a disease associated with ocular neovascularisation.

The present study is therefore of great importance as it has enabled the researchers to identify the anti-angiogenic activity, i.e. a much sought-after property in medical practices, from a marine organism. The purified fraction(s) need to be assayed in the experiments on *in vivo* models. It also has to be subjected to further analysis using the tools of proteomics and gene arrays. At present, various experiments are designed in order to take these observations further so that the researchers could, without long delay, avail of a useful and reliable substance/ molecule in the attempts to contain malignant growth.

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