Screening for Anti-Inflammatory Potential of Marine Actinomycetes

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Abstract
This work was carried out with marine actinomycetes since they were considered to be a potent source for the production of pharmaceutically important secondary metabolites. Sea water was collected at 700 m deep from Kovalam beach and subjected to serial dilution. The seventh dilution was inoculated on ISP 1 medium and incubated for 7 days at 28°C and sub-cultured for good sporulation. The potent actinomycetes were characterized by observing colour and appearance of colonies. After 7 days, the secondary metabolites were extracted using ethyl acetate solvent and subjected to physiochemical analysis which reported the presence of tannins, flavonoids, quinones and phenols. Antioxidant potential of extract was evaluated by means of DPPH assay using standard procedures.

The results showed 58% inhibition of DPPH radicals at maximum concentration of 100µg/ml. Ascorbic acid was the positive control used. The anti-inflammatory potential of extract was evaluated by means of HRBC assay. The extract showed dose-dependent protection and inhibition of haemolysis. The positive control used was diclofenac. The results showed 99.5% protection against haemolysis. The extracted bioactive compounds has shown good antioxidant and anti-inflammatory properties and further research is in progress to produce bioactive compounds in large quantities.

Keywords: Marine actinomycetes, ISP 1 Medium, Ethyl acetate solvent, DPPH assay, HRBC assay, Anti-inflammation potential.

Introduction

The marine ecosystem of the Indian peninsula is rich in microbial diversity and has a highly complex environment and has been proved to be a good habitat of diverse assemblage of microorganisms due to extreme variations in temperature and salinity. The natural products reported from microbial sources are approximately 32,500 including about 1000 derived from marine microbes[1]. Marine ecosystems are different from aquatic ecosystems because of their unique features including the presence of dissolved compounds in sea water particularly sodium chloride. It is derived that marine microorganisms differ in characteristics from those of terrestrial counterparts and hence produce different types of bioactive compounds[2].

Actinomycetes are filamentous gram-positive bacteria[3] with heterogenous nature which is the source for its taxonomic instability. They form branching filaments or hyphae and asexual spores. They branch out forming a network of hyphae growing both on the surface and under-surface of the agar when grown on an agar-surface[4]. They are abundant in soil and are responsible for digestion of resistance carbohydrates such as chitin and cellulose. They produce many antibiotics such as amphotericin, nystatin, erythromycin, vancomycin, neomycin etc.

They play a major role in production of bio-emulsifiers because they are effective at extreme conditions of pH, salinity and temperature[5]. They play an important role in bioremediation and enzyme inhibition. They are involved in all the processes that contribute to soil fertility.

The actinomycetes are considered to be the most potent source for the production of secondary metabolites and play a special role in antibiotic production. They produce unique and novel secondary metabolites which play an extensive role in the pharmaceutical and medical industry. It is postulated that each strain of actinomycetes has genetic potential ability to produce 10-20 secondary metabolites[6]. A large number of bioactive molecules have been produced from the genus streptomyces alone[7]. They remain unchallenged among other biological groups till date.

Secondary metabolites comprise various chemical moieties such as amino acid derivates and sugar and their biosynthesis is catalyzed by a number of enzymes which are usually encoded by genes[8]. Marine actinomycetes may have different characteristic from terrestrial actinomycetes since environmental conditions of the sea are extremely different from terrestrial conditions and therefore might produce novel bioactive compounds and new antibiotics. This research study highlights the extraction of secondary metabolites from marine actinomycetes for determining its activity on anti-inflammation.

Material and Methods

Sample collection: Sea water was collected from Kovalam beach, 40 km south of Chennai on the east coast road. It was collected at 700 m deep to ensure presence of high amounts of actinomycetes. The sample was stored in sterile bottles and transferred to the laboratory.

Isolation and inoculation of actinomycetes: The sample was subjected to serial dilution. 1ml of the sample was suspended in 10ml of distilled water. The serial dilution

technique was performed and the range of dilution is 10^{-1} to 10^{-7}. The dilution 10^{-7} was selected for isolating actinomycetes. 0.1ml of the dilution was inoculated in the medium. The medium used for isolation of actinomycetes was international streptomyces project (ISP no. 1 media). The medium was prepared according to the composition and sterilized at 121°C in 15 lbs pressure for 15 minutes. The medium was cooled and was supplemented with fluconazole (5µg/ml) to avoid fungal contamination.

The procedure was performed in a laminar air flow chamber with high precaution. A sterile L rod was used for inoculation. After solidification of media, the plates were labeled and inoculated by transferring 100µl of suspension over the surface of ISP medium by spread plate method and the plates were incubated at 28±2 °C for seven days[9].

Secondary metabolite extraction: Solvent extraction method was employed to recover secondary metabolites in the pure form. The solvent ethyl acetate was added to the actinomycetes strains scrapped off from the culture plates in the ratio 1:3 and was shaken vigorously for 20 minutes for complete extraction. Ethyl acetate is the chosen solvent because it tends to pull more of desired product from aqueous phase to organic layer. This was allowed to soak for one day and evaporated to dryness after filtration using blotting paper. It was air dried until powder consistency is obtained.

Phytochemical analysis: The extracts were subjected to phytochemical analysis, a preliminary qualitative phytochemical screening technique to determine the presence of various secondary metabolites according to the procedure. The analysis was done for detecting the presence or absence of phytonutrients such as carbohydrates, tannins, saponins, flavonoids, alkaloids, glycosides, cardiac glycosides, terpenoids, phenols, steroids, phlobatannins and anthraquinones.

Anti-oxidant activity - estimation of radical scavenging activity using DPPH assay: DPPH (1,1-diphenyl 2-picrylhydrazyl) was used to determine the free radical scavenging activity of each fraction taken. The stock solution was prepared in concentration of 10mg/ml. Different concentrations (200µl, 400µl, 600µl, 800µl, 1000µl) of the extract of sample were added at an equal volume to methanolic solution of DPPH (0.1mM). This reaction mixture is incubated for 30 minutes in a dark environment at room temperature and the absorbance was recorded at 517nm.

The experiment was repeated three times using ascorbic acid as standard control and the absorbance of the DPPH control (without extract/standard) was noted[10]. The annihilation activity of free radicals was calculated in percentage inhibition according to the following formula:

$$[\text{Absorbance of control} - \text{absorbance of test sample} \div \text{absorbance of control}] \times 100$$

Anti-inflammatory activity - estimation of anti-inflammatory activity using HRBC assay: Hypotonicity induced human red blood cell (HRBC) membrane stabilization method. Blood was collected from healthy volunteers and it was mixed with equal volume of sample solution (Table 4) and centrifuged with isosaline[11]. 1.0mL of test sample was taken in different concentrations (200µL, 400µL, 600µL, 800 µL, 1000µL) in 1ml of 0.2M phosphate buffer, 0.5mL of 10% HRBC suspension and 0.5ml of 0.25% hyposaline. It was then incubated at 37°C for 30 minutes and centrifuged at 3,000 rpm for 20 minutes.

The hemoglobin content of the supernatant solution was estimated at 560nm spectrophotometrically[12]. Diclofenac was used as standard and a control was prepared by distilled water instead of hyposaline to produce 100 % haemolysis without samples. The percentage of HRBC haemolysis and membrane stabilization or protection was calculated by using the following formula:

$$\text{Percentage of haemolysis} = \frac{\text{optical density of test sample}}{\text{optical density of control}} \times 100$$

$$\text{Percentage of protection} = 100 - \left(\frac{\text{optical density of test sample}}{\text{optical density of control}}\right) \times 100$$

Results and Discussion

Isolation and inoculation of actinomycetes: Serial dilution was performed to reduce a dense culture of cells and to produce concentrations which are easy to use since each dilution will reduce the concentration of bacteria by a specific amount. A tenfold serial dilution is usually done. The range of dilution was 10^{-7} and 0.1ml of the seventh dilution was inoculated in the freshly prepared medium (ISP 1 medium) which was prepared according to appropriate concentrations as specified in table 1.

Table 1
ISP 1 Medium composition

Component	Quantity
Casein	0.15 g
Yeast	0.25 g
Agar	2.5 g
Distilled water	50ml

The medium was supplemented with fluconazole (5µg/ml) to prevent fungal contamination. The media preparation was done in a laminar airflow chamber to prevent contamination of media. The media was autoclaved for 15 minutes and allowed to solidify. After media was solidified, the suspension was inoculated on the medium using spread plate method and was incubated for seven days at 28°C[13]. Figure 1 shows the culture of actinomycetes which are grown well and have good sporulation. The quadrant streaking is done to show the thinning of colonies and to isolate pure culture of our interest and subject it to sub culturing.

After incubation for seven days, good sporulation was observed which was further characterized morphologically to determine the presence of actinomycetes. The characteristics of actinomycetes such as whitish pin point, powdery and dry colonies with clear zone of inhibition were observed. These colonies were sub-cultured in ISP 1 medium to obtain pure cultures. After incubation for seven days at 28°C, good sporulation of actinomycetes without fungal contamination was observed in the plate in figure 2.

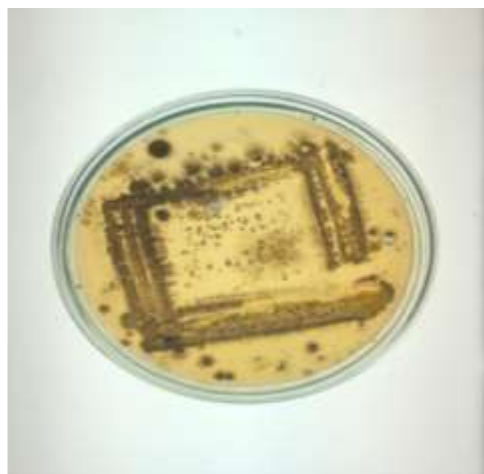

Figure 1: Mother culture of marine actinomycetes

Extraction and characterization of phytochemicals: The extraction was done by ethyl acetate solvent. This solvent was chosen because it has the ability to pull more of metabolites from the aqueous phase into the organic phase. After 24 hours inhibition, the actinomycetes were taken in by the solvent resulting in a mat layer.

This mixture was stored in an incubator at 28°C for future use. Phytochemical analysis was done according to the standard procedure to determine the presence or absence of various secondary metabolites in the actinomycetes isolates and the results are provided in table 2.

Figure 2: Sub culture of marine actinomycetes on ISP 1 Medium at 28°C (after incubation for 7 days)

Evaluation of anti-oxidant activity: In recent years, much attention has been given to natural oxidants and their benefits in health. Several methods are employed to assess and determine the anti-oxidant activity of bioactive compounds. Free radical scavenging assay using 1,1, diphenyl-2-picrylhydrazyl (DPPH) is an easy, rapid and sensitive method for the anti-oxidant screening. A stable diamagnetic molecule can be formed by DPPH which is a stable free radical. DPPH radical obtains one more electron and the absorbance decreases in the presence of an anti-oxidant. In this activity, the scavenging activity of extracts was found to be dose dependant.

The scavenging activity was more when the concentration was higher. The results show 58% inhibition of DPPH radicals at maximum concentration of 100µg/ml which shows that the extracts have the ability of proton-donating ability and could serve as free radical inhibitors or scavengers.

Table 2
Results of phytochemical analysis

S.N.	Phytochemical Tests	Results
1.	CARBOHYDRATES TEST	ABSENT
2.	TANNINS TEST	PRESENT
3.	SAPONINS TEST	ABSENT
4.	FLAVANOIDS TEST	PRESENT
5.	ALKALOIDS TEST	ABSENT
6.	QUINONES TEST	PRESENT
7.	GLYCOSIDES TEST	ABSENT
8.	CARDIAC GLYCOSIDES	ABSENT
9.	TERPENOIDS	ABSENT
10.	PHENOLS	PRESENT
11.	COUMARINS	PRESENT
12.	STEROIDS	ABSENT
13.	PHLOBATANNINS	ABSENT
14.	ANTHRAQUINONES	ABSENT

Even though the DPPH radical scavenging activities of the extracts were less than that of ascorbic acid, it can possibly act as primary anti-oxidants. Table 3 shows the evaluation of antioxidant activity. The different concentrations of extract with equal volume of DPPH solution were spectrophotometrically observed to obtain the OD values for both the extract and positive control which is ascorbic acid. The readings were tabulated to obtain graphical results.

Figure 3 shows the graphical representation of anti oxidant activity of the extracts. As the concentration of extract increased, an increase in percentage of inhibition of oxidation was observed. Though the values were not in correspondence with the standard control values, the obtained values were satisfactory and showed antioxidant potential.

Evaluation of anti-inflammatory activity: Red blood cells are subjected to haemolysis when they are exposed to injurious substances such as hypotonic medium which results in lysis of its membrane[14]. The secondary metabolite extract of marine actinomycetes significantly protects the human blood erythrocyte membrane against lysis.

The extract showed dose-dependent protection and inhibition of haemolysis. As the concentration of extract increased, the percentage of haemolysis decreased and as the concentration of extract increased, the percentage of protection against haemolysis increased providing stabilization of the membrane. Diclofenac was used as a positive control since it showed a significant protection of RBC against the damaging effect of hypotonic solution. Highest concentration of extract showed 0.59 percentage of haemolysis whereas the highest concentration showed 99.40% of protection of cell membrane, thereby decreasing the inflammation.

Figure 4 shows the graphical representation of percentage of haemolysis which was obtained from results provided in table 4. Diclofenac is the standard control used as it is administered for patients with inflammation. This shows that the extract has potential to decrease the rupture of blood cells which is a sign of anti-inflammation.

Figure 5 shows the graphical representation of comparison of percentage of protection with positive control which was obtained from the results provided in table 5. As the concentration of extract increased, protection of cell membrane against hypotonic solution was increased but percentage of protection is decreased even after increasing the concentration of the sample (800 µg/ml).

Table 3
Evaluation of antioxidant activity of different concentrations of extract

Concentration of sample (µg/ml)	Percentage of inhibition	Positive control
200	30.64	93.7
400	42.22	94.8
600	45.96	97.3
800	55.66	98.2
1000	56.21	99.5

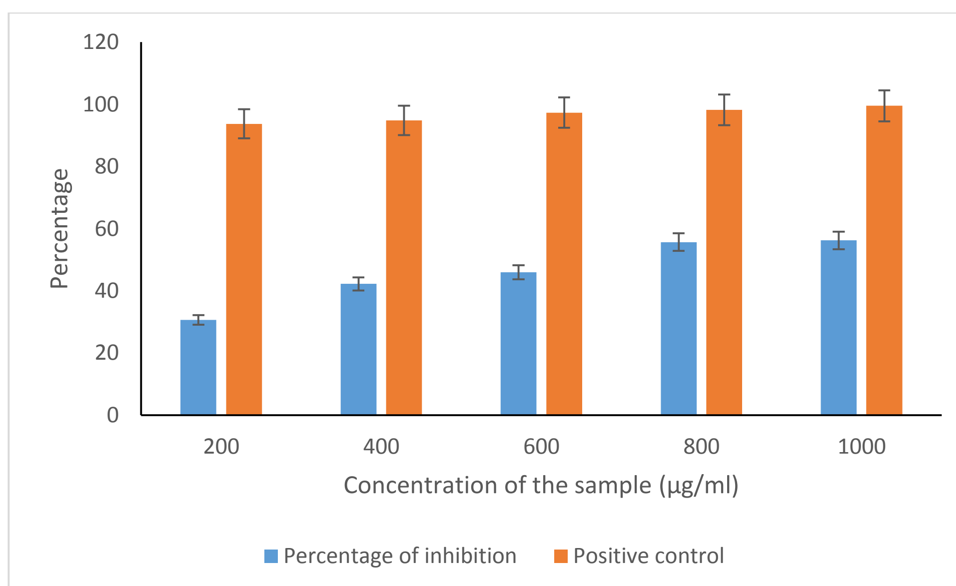

Figure 3: Comparison of scavenging effect of different concentrations of extract with standard ascorbic acid, an increase in percentage of inhibition was observed.

Table 4
Evaluation of percentage of protection of increasing concentrations of extract which showed the increase in protection of cell membrane stability

Concentration of sample(µg/ml)	Percentage of protection	Positive control
200	84.55	96
400	90.49	98
600	95.73	97
800	96.18	99
1000	99.40	99

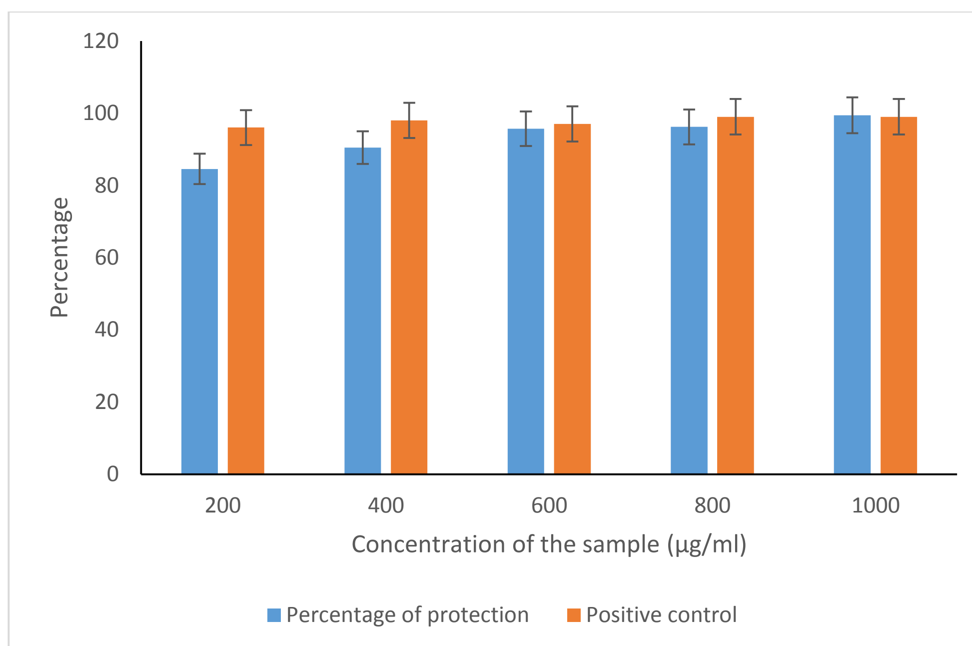

Figure 4: Comparison of percentage of protection of different concentration of extract with Diclofenac (positive control)

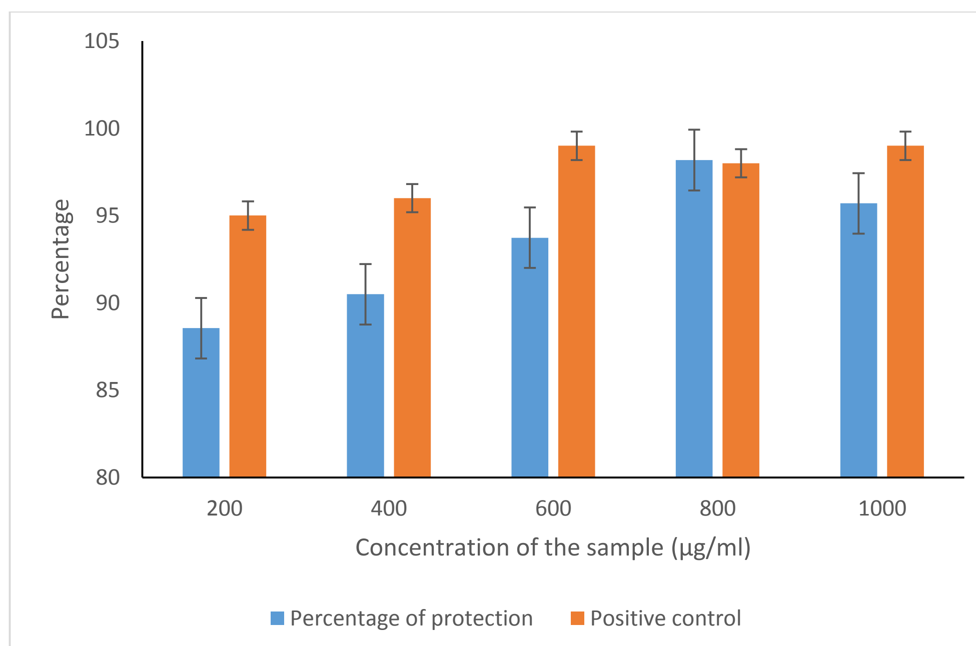

Figure 5: Comparison of percentage of protection of different concentration of extract with Diclofenac (positive control)

Table 5
Evaluation of percentage of protection of increasing concentrations of extract which showed the increase in protection of cell membrane stability

Concentration of sample(µg/ml)	Percentage of protection	Positive control
200	88.55	95
400	90.49	96
600	93.73	99
800	98.18	98
1000	95.70	99

Though the results were not as efficient as Diclofenac control, the values obtained for the extract were good and the percentage of protection was observed to be satisfactory. The results obtained thus demonstrate that secondary metabolites of marine actinomycetes can significantly and dose-dependently inhibit HRBC haemolysis. Though the percentages are less than the results obtained by diclofenac control, the extract has good membrane stability and hence good anti-inflammatory activities.

Conclusion

Marine environments are complex in nature because they occur in environments with extreme variation in pressure, salinity and temperature and have varied group of life forms. They produce different kind of metabolites, which could not be produced by the terrestrial microorganisms, since they have developed exceptional metabolic and physiological capabilities to be able to survive in such intense habitats. Extensive research has revealed that actinomycetes are prolific sources of novel metabolites. In the present study, sea water was collected from kovalam beach and was subjected to serial dilution and inoculated in ISP 1 medium which was supplemented with fluconazole. After good sporulation was seen, it was sub-cultured with the same medium and secondary metabolites were extracted with ethyl acetate solvent.

Qualitative phytochemical analysis showed the presence of secondary metabolites such as tannins, flavonoids, phenols, quinones and coumarins. Antioxidant activity of the compound was done using DPPH assay which is a rapid and easy method. The scavenging activity was dose-dependent in this study. The results showed 58% inhibition of DPPH radicals at maximum concentration of 100µg/ml. Ascorbic acid was the positive control used. Though the scavenging ability of the extract was less than that of positive control, it can act as primary antioxidant. Red blood cells are subjected to haemolysis when they are exposed to injurious substances such as hypotonic medium which results in lysis of its membrane. The secondary metabolite extract of marine actinomycetes significantly protects the human blood erythrocyte membrane against lysis.

The extract showed dose-dependent protection and inhibition of haemolysis. The positive control used was diclofenac. The result showed that the percentage of protection is less than the results obtained by diclofenac control, the extract has good membrane stability, hence good anti-inflammatory activities are obtained. Secondary metabolites of marine actinomycetes can significantly and dose-dependently inhibit HRBC haemolysis. The extracted bioactive compounds have shown good antioxidant and anti-inflammatory properties and further research is in progress to produce bioactive compounds in large quantities.

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