# Screening Of Marine Bacteria For Pharmacological Activities

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**Abstract**: The symbiotic and associated four marine bacteria BR1 (Flavobacterium sp.) isolated from Barnacle Balanus amphitrite,EM13 (Micrococus sp.) from Seaweed Enteromorpha compressa,PC4 (Alcaligenes sp.) from Ascidian Polyclinum constellatum and SW12 (Bacillus sp.) from seawater were cultured and extracted for pharmacological activities. The ethyl acetate extracts of these marine bacterial culture supernatants were screened for pharmacological activities such as Anti inflammatory, Analgesic and CNS depressant activities using experimental animal model. In this study,SW12 exhibited high activity for both Anti inflammatory and Analgesic. Especially which exhibited highest analgesic activity than standard drug pethidine. Another one PC4 showed highest analgesic activity similar to standard drug. Other two extracts EM13 and BR1 showed high activity in CNS depressant. Based on the result, SW12 is a highly potent strain, it may produce novel compound for pharmacological drug.

**Keywords:** Analgesic activity, Animal model, Anti inflammatory, CNS depressant activity, Ethyl acetate extracts,Marine bacteria and Pharmacological drug.

## 1 INTRODUCTION

In the last 25 years, the marine organisms such as algae, invertebrates and microbes have provided key structures and compounds, which proved their potential in various areas, especially in medical field as new therapeutic agents for a variety of diseases [1].The vast potential of marine organism as source of bioactive metabolites that may be directly utilized as drugs or serve as lead structures for drug development started in the late 1960s.The discovery of various quantities of prostaglandins, which had just been discovered as important mediators involved in inflammatory diseases, fever and pain ,in gorgonian *Plexaura homomalla* introduced by [2].It is usually considered as take off point of the drug discovery from the sea , however that unusual nucleosides isolated from marine sponges already in the 1950s.Which served as lead structures for the development of recent commercially important anti-viral drugs such as ara-A and the anticancer drug for leukemia, ara-C. The interest of the pharmaceutical and well developed chemical companies for the marine biotechnology is mostly driven by nature's diversity in novel molecular structures with unexpected yet beneficial functions and various activities. Those may lead to novel pharmaceuticals with anti-cancer, anti-inflammatory and antibiotic properties [3].Some represent new classes of pharmacological agents that exhibit mechanism of action different from currently used drugs. For example, the Pseudopterosins are diterpene glycosides, which was having potent anti inflammatory and analgesic agents, that was isolated from the gorgonian, *Pseudopterogeogia elisabethae* [4].

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A wide range of useful drugs including antibiotics, analgesics, anti-inflammatory, anticoagulants, CNS depressants, antipyretics agents etc.have been isolated from marine organisms [5]. Currently, only few marine derived products are in the market and several of them are in clinical trials [6].

### Marine Bacteria

Microorganism have had a major effect on the development of medical science, since the discovery that they not only the cause of infection, but also they produce organic compounds, that can cure infection and help to treat a variety of non infectious diseases. Since the original discovery of penicillin in 1929, nearly 50,000 natural products have been discovered from microorganisms [7].The research for the marine microorganisms that they provide a potent source of biomedically useful compounds obtained from studies showing that marine bacteria produce antimicrobial agents [8]. Natural products from microbes, especially the associated organisms, remain one of the most important sources of lead compounds for the pharmaceutical industry and have been synthesized and others serve as leads in the synthesis of bioactive analogs [9]. The marine bacteria were extremely difficult to isolate and culture, however, that bacteria are now known to be capable of producing unusual bioactive natural products that are not observed from terrestrial sources [10],[11]. For example Fenical group isolated a novel class of antiviral and cytotoxic macrolides from a deep sea marine bacterium [12].The discovery that several well known marine toxins are produced by marine microorganisms, for example the potent neurotoxin tetrodoxin has been known for years and was considered to be produced by puffer fish of the family Tetraodontidae, The research result that tetrodotoxin is produced by many marine bacteria [13]. The study of marine bacteria has also led to the realization that microorganisms form specific symbiotic relationship with marine macroorganisms, which introduces real uncertainty about the exact metabolic origin of the bioactive compounds that have been isolated from such organisms [14],[10].The potential value of some selected secondary metabolites, all obtained from sponges and their associated microorganisms. Example of compounds that are already used in medical purpose or are being considered as

lead structures acting as cytostatic and anti inflammatory agent. Many studies on bioactive compounds from marine bacteria exhibiting anti tumour, antiviral, antifungal and antibacterial activities have been reported worldwide. The research for anti-inflammatory and analgesic agent in modern times was commercialized by the introduction of salicin for the treatment of inflammatory swelling due to rheumatic fever and rheumatoid arthritis. The severe side effects of steroidal and non steroidal anti inflammatory drugs have lead to search for new anti inflammatory agents. The aim of the study was to evaluate the anti inflammatory, analgesic and CNS depressant activity of the crude ethyl acetate extracts of four potential marine bacteria BR1 (*Flavobacterium sp.*), EM13 (*Micrococus sp.*), PC4 (*Alcaligenes sp.*) and SW12 (*Bacillus sp*.)

## 2 Materials and Methods

The extract of marine bacterial culture BR1 (*Flavobacterium sp.*) isolated from Barnacle *Balanus amphitrite*, EM13 (*Micrococus sp.*) from Seaweed *Enteromorpha compressa*, PC4 (*Alcaligenes sp.*) from Ascidian *Polyclinum constellatum* and SW12 (*Bacillus sp*.) broth were made by following the method of [15], [16].The sample of seawater , seaweed and invertebrate species were collected at low tide. For isolation of epiphytic bacteria, the sample was washed two to three times in sterile seawater to remove loosely attached bacteria. The sample was then placed in another 10ml of sterile sea water and vortexed to remove more tightly bound epiphytes. Cell suspensions obtained in this way were used to inoculate agar plates of 50 percent marine agar (Zobell agar and seawater agar, Himedia, Mumbai).The plates were then incubated (25$^{o}$c,5 days) and representative colonies were picked and isolated by successive restreaking. The isolated strains were stored in marine agar slants at 4$^{o}$c. Isolated bacteria were inoculated into the separate conical flask containing seawater broth .They were inoculated in orbital shaker incubator (250 rpm) at 30$^{o}$c for 7 days. After incubation each sample was centrifuged at 13,000 rpm for 10 minutes to pellet the bacterial cells. The supernatant was filtered through a 0,22mm filter (Millipore) to remove the bacterial cells, after that transferred into sterile tubes. The supernatant was extracted with equal volume of ethyl acetate and sonigated for 10 minutes for mixing. Then the ethyl acetate extract was transferred to sterile round bottom flask for removal of solvent. The concentrated mixture was transferred to sterile Eppendorf tubes (1.5 ml) and used for the pharmacological activities.

### Animals

Albino rats (HA strain) of 150-200 g were obtained from laboratory Animal Resource Section, Arulmigu Kalasalingam College of Pharmacy, Pharmacology Research Lab at Anandnagar ,Srivilliputhur. The animals were kept in polypropylene cages in an air conditioned area at 25+2$^{0}$c in 10:14 hr light dark cycle. They were provided with Amrut brand balanced feed and tap water *ad libitum.* The extract of marine bacteria were devoid of any mortality or change in behaviour upto 1g/Kg orally in albino rats. Based on this observation maximum dose of 200µg/kg i-p were used for acute treatment in following experiments.

### Experimental methods

#### Anti-inflammatory activity-Carrageenan induced Rat paw oedema method

Thirty six rats are divided into six rats each for various treatments as shown in table 1.Subsequently 30 min after above treatment 0.1ml of 1% carrageenan was injected subcutaneously into the planter region of right hind paw to induce oedema. The paw volume was measured initially and at 1,2,3 and 4hour after carrageenan injection using phythesmographic method [17],[18]. Percentage of inflammation was calculated for comparision.

#### Analgesic activity- Hot plate reaction time method

Analgesic activity was tested in Albino swiss mice weighing between 20-25 g with six numbers of animals in each group. They were kept in polypropylene cages and maintained on balanced ration with free access to clean drinking water. The central analgesic action of marine bacterial extracts were assessed by hot plate in mice. Healthy male swiss albino mice weighing 20-25 g was divided into six groups of each six animals. Group I considered as control, Group II received standard drug pethadine (5mg/kg) i.p., while group III,IV,V and VI received ethyl acetate extracts of marine bacteria at a dose of 200µg/kg (Table 2).The animals were placed on the hot plate maintained at 55-56$^{o}$C temperature and the time until either paw licking or jumping was noted [19].

#### CNS Depressant activity: Effect of pentobarbitone sodium induced sleeping time

Healthy adult male albino swiss mice weighing 20-25 g were selected and fasted for 24 hours prior to experiment. They divided into six groups, each with six animals and their basal activity score were recorded and numbered. The test compounds were administered intra peritonially at a dose of 100g/kg. The control group of animals were given only the vehicle. After 30 minutes, Pentobarbitone sodium was administered intraperitonially to all group of animals at a dose of 35mg/kg. The time of administration of test compounds and pentobarbitone sodium, the time of loss and gain of righting reflex were recorded in all the group of test animals and percentage effect on pentobarbitone sodium induced sleeping time by the test compounds were calculated using this formula (1), considering righting reflex in control an 100 percent.

$$\%\text{effect} = \frac{\text{Average duration of loss of righting reflex in test group}}{\text{Average duration of loss of righting reflex in control group}} \quad (1)$$

### Statistical Analysis

The values were expressed as mean $\pm$ SD of four replicates for each experiment. The data were analysis using Student's t-test and $p<0.05$ was considered as significant.

## Results

### Anti inflammatory activity

The effect of BR1, PC4, EM13 and SW12 extracts on carrageenan induced oedema is shown in Table 1.The extracts as well as indomethacin showed antiphleogestic activity. This anti inflammatory activity was found to be statistically significant at dose of 200 µg/kg of crude bacterial extracts. The anti inflammatory activity of indomethacin a standard reference

drug was also found to be significant.SW12 exhibited high activity at dose of 200 µg/kg. EM13 and PC4 were showed moderate activity in the same dose. But BR1 gave poor activity (see Table 1).

***Table -1***

*Acute anti inflammatory activity of crude ethyl acetate bacterial extracts on carrageenan induced –Rat paw oedema*

| Drug | Dose mg/kg | Percentage of inflammation at time (hours) | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| Ctrl | -- | 38.61±4.56 | 80.86±2.92 | 96.76±6.26 | 110.82±5.02 |
| Std. | 10 | 12.25±2.92* | 20.28±3.85* | 26.27±3.22* | 33.60±3.42* |
| BR1 | 200 µ | 33.26±5.45 | 62.38±4.20 | 91.38±3.20 | 103.86±5.66 |
| PC4 | 200 µ | 17.38±3.48* | 51.68±3.80* | 83.32±5.12* | 91.21±3.98* |
| EM13 | 200 µ | 15.62±2.98* | 45.62±2.92* | 52.82±3.62* | 88.21±2.86* |
| SW12 | 200 µ | 18.72±3.38* | 56.26±2.82* | 51.26±2.80* | 57.42±3.68* |

*Ctrl= Control; Std.=Indomethacin;*
**P<0.05 as compared to control group.*
*Values are mean +SEM; n=6 in each group.*

### Analgesic activity-Hot plate method

At 0 minute the reaction time of mice when placed on the hot plate ranged from 8.65 ±1.62sec. to 9.32 ±1.06sec.The reaction time was almost constant in control animal.But marine bacterial extract treated animals particularly SW12 showed increase in the reaction time dependent manner than standard drug pethidine at dose of 200 µg/kg. The peak analgesic activity was observed 2hrs after administration of marine bacterial extracts and the analgesic activity persisted for 3 hrs only. The standard narcotic analgesic pethidine (5mg/kg) exhibited analgesia for 2hrs only. Next extract PC4 exhibited nearly same activity to standard drug. Other extracts showed moderate activity(see Table 2).

**Table- 2**

Effect of crude ethyl acetate bacterial extracts on Thermic stimulus induced pain (hot plate test) in mice.

| Drug | Dose mg/kg | Reaction time in seconds at time (hours) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 0.5 | 1 | 2 | 3 |
| Ctrl | -- | 8.65±1.62 | 8.79±1.08 | 8.10±0.68 | 13.20±1.21 | 10.82±1.36 |
| Std | 5 | 9.32±1.06 | 10.84±1.37 | 15.32±2.09 | 18.44±1.52 | 13.88±0.94 |
| BR1 | 200 µ | 9.25±1.52 | 7.22±0.64 | 8.98±0.73 | 14.22±1.20 | 11.86±1.20 |
| PC4 | 200 µ | 8.49±1.26 | 8.26±1.22 | 10.26±1.72 | 16.20±1.06 | 13.76±2.08 |
| EM13 | 200 µ | 8.42±1.72 | 8.16±1.21 | 11.26±1.81 | 13.90±1.06 | 12.82±2.06 |
| SW12 | 200 µ | 8.51±0.83 | 10.47±1.05 | 10.12±0.64 | 16.89±1.66 | 16.26±1.65 |

*Ctrl= Control; Std.=pethadine;*

### CNS depressant activity

The result of effect on pentobarbitone sodium induced narcosis showed that all test compounds potentiated pentobarbitone sodium induced sleeping time from 208 per cent to 281 percent. The crude ethyl acetate extract of EM13 and BR1showed more activity with a potentiation of 281 percent and 278 per cent. The crude extract PC4 and SW12 were found to be next in the order of potentiation of pentobarbitone sodium induced sleeping time (see Table 3).

***Table- 3***

*Effect of crude ethyl acetate bacterial extracts on Pentobarbitone sodium induced sleeping time*

| S.No. | Compound | Sleeping | Percent Effect |
|---|---|---|---|
| 1. | PC4 | 65.00 | 208.00 |
| 2. | BR1 | 78.00 | 278.00 |
| 3. | SW12 | 67.00 | 210.00 |
| 4. | EM13 | 80.00 | 281.00 |
| 5. | Control | 40.00 | 100.00 |

## DISCUSSION

### Anti inflammatory activity

The intraplanter injection of the hind paw by carrageenan induced a progressive oedema; this model is useful to detect anti inflammatory activity of different agents. Inflammation induced by carrageenan involves three distinct phases of the release of the mediator, including serotonin and histamine in the first phase (0-2hours). Kinins in the second phase (3 hrs) and prostaglandin in the third phase (74 hrs).So, In the present study, the observation was made 4 hours of inflammation. The crude ethyl acetate extracts of EM13,PC4,SW12 and BR1 significantly inhibited paw oedema respectively. To ascertain the effect of those 4 ethyl acetate bacterial extracts ,as the activities of the mediator 1 litre was tested an inflammation induced by histamine and serotonin characterized by vascular permeability. It was observed that these 4 bacterial extracts were capable of inhibiting oedema induced by histamine and serotonin. The marine microbes have been the source of potential antimicrobial, cytotoxic and pharmacological activities. The alkaloid oxepinal isolated from marine fungus *Acremonium sp*[20] and the scytonemin pigment ,isolated from marine cyanobacteria [21] are the typical examples of marine microbial compounds showing anti inflammatory activity. The associated microbes were the actual producers of the active metabolites was substantiated by the pseudopterosins, diterpene glycosides isolated from the Caribbean sea whip *Pseudopterogorgia elisabethae*, which was shown to possess anti inflammatory and analgesic activities [4],was reported to originate from the dinoflagellate symbiont *Sympoidinium sp*., localized within the tissues of the sea whip [22]. In the present investigation ,the higher anti inflammatory activity was observed in EM13 crude ethylacetate bacterial extracts followed by PC4,SW12 and BR1.It has been reported that the second phase of oedema is sensitive to both clinically usefull steroidal and non steroidal anti inflammatory agents. It has been reported that most anti inflammatory agents might produce gastric irritation and even ulceration with prolonged use. In this study, the bacterial extracts significantly exhibited the inflammation of first two phases implied that the extracts might be clinically useful for anti inflammation, if the active metabolite of these extracts characterized.

### Analgesic activity

The results of hot plate test indicated a significant increase in reaction time at 2 and 3 hrs with 200mg/kg especially higher analgesic activity exhibited by SW12 (*Seawater*) and PC4 (*Polyclinum consellatum*).The analgesic activity of other bacterial extracts (EM13 and BR1) were showed moderate as compared to potent inhibitory activity of pethidine. Pethidine offers release from pain by suppressing the formation of pain inducing substances in the peripheral tissues, where prostaglandins and bradykinin were suggested to play an important role in the pain process. Therefore it is likely that potential bacterial extracts suppress the formation of these substances of antagonize the action of these substances and thus exerts its analgesic activity in hot plate test suggesting its central analgesic activity. The hot plate method was first described by [23].This test has been found to be suitable for evaluation of centrally but not peripherally acting analgesic. In order to distinguish between the central and peripheral analgesic action of ethylacetate extracts of EM13,PC4,SW12 and BR1.Hot plate reaction time method response in mice was used to examine the effect. This method is not only simple and reliable, but also affords time dependent evaluation of peripheral type analgesic action. The marine bacterial extracts may act by inhibiting the cyclooxygenase enzyme activity which is responsible for pain sensation.

### CNS depressant activity

The locomotor activity of the animal was reduced by the extracts of the bacterial symbionts. In control, the activity was found to be negligible. The standard drug diazepam treated animal exhibited nearly 60% reduction in locomotor activity. The 200mg/kg dose of the crude extract of EM13 exhibited a reduction of 80% of the locomotor activity. In the experiment to evaluate the pentobarbitone sodium induced time, the crude extract of bacteria not only reduced the time of onset of pentobarbitone sodium induced sleeping time but also prolonged sleeping time. In the present study, ethylacetate extracts of four different bacterial isolates (EM13,PC4,SW12 and BR1) exhibited CNS depressant activity in mice tested by actophotometer and pentobarbitone sodium induced time. The ethylacetate extract of the bacterial symbiont EM13 from *Enteromorpha compressa* and BR1 from Barnacle *Balanus amphitrite* potentiated the activity of pentobarbitone sodium induced time. [24] studied the CNS depressant activity of Ascidian *Distalpia nathensis* . The locomotor activity of the rat was greatly reduced by the extracts and the activity was dose dependent with higher dose of higher depressant activity [5]. The possible mechanism of CNS depressant activity by the bacterial extract may be attributed to the enhancement of Gama Amino Butyric Acid (GABA) in brain.

## CONCLUSION

Based on the results of the present study it can be conclude that the ethylacetate extracts of marine bacterial strains EM13, PC4, SW12 and BR1 have potential anti inflammatory activity against acute inflammation, CNS Depressant and also have analgesic activity. Hence, further purification of these extracts may lead to the discovery of novel pharmaceutical potential compounds.

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