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Anti Inflammatory, Antinociceptive and Central Nervous System Depressant Activities of Marine Bacterial Extracts

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Abstract: The main objective of this study is to isolate the bacterial strains which are producing biomedicinally relevant secondary metabolites. To achieve this, the ethyl acetate extracts of four marine bacterial strains BR1, PC4, EM13 and EM14 which were isolated from *Balanus amphitrite* (barnacle), *Polyclinum constellatum* (ascidian) and *Enteromorpha compressa* (Seaweed), respectively subjected to study the anti inflammatory, analgesic and central nervous system depressant activities. Anti inflammatory activity was studied by carragennan induced rat paw edema model. Though the results were significant ($p<0.05$) for all the four bacterial extracts the more effective anti-inflammatory activity was exhibited by EM13 and EM14 (range between 20-59% of inhibition). Interestingly EM13 inhibited early phases, whereas EM14 inhibited the later phases of inflammation. These two extracts produced the same effect on analgesic activity which was studied by using hotplate test. However, the ethyl acetate extracts of EM13 and BR1 showed remarkable reduction in locomotor activity and prolongation of phenobarbitone sodium induced sleeping time that demonstrated the significant CNS depressant activity. The experimental data identified that the strains EM13, EM14 and BR1 contain potential pharmacologically active compounds and suggested that to further isolation and characterization of active principles and phylogenetic identification of the epibiotic bacterial strains. The present study evidenced that the bacteria associated with marine organisms are the potential sources of pharmacologically active natural products.

Key words: Marine bacteria, anti-inflammatory, antinociceptive, CNS depressant activities, ascidian, barnacle, seaweeds

INTRODUCTION

Emergence of new diseases and increasing incidence of bacterial resistance has necessitated the mankind to look constantly of new alternative source of medicines. Marine animals and their associated micro organisms produce unique compounds of biomedical importance which are not found in terrestrial environment (Carte, 1996). The vast diversity of marine organisms and habitats encompass a wide variety of chemical classes of terpenes, polyketides, acetogenins, peptides and alkaloids varying structures representing biosynthetic schemes of stunning variety (Wright, 1998). Over the past 4 decades marine organisms have been the focus of a worldwide effort for the discovery of novel natural products. More than 12,000 novel chemicals with hundreds of new compounds still being discovered every year from marine invertebrates and their associated micro organisms (Donia and

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Hamann, 2003). These metabolites have been developed or currently being tested as antibiotics, analgesic, anti inflammatory, CNS depressant, anticancer, antiviral agents. One of the major obstacles of the marine natural products translated into clinical trails is supply issue. The concentrations of many highly active compounds are often minute (10^{-9} of wet weight) in organisms (Proskch *et al*., 2002). So, scientists have been looked for alternative source of marine natural products without extinction of the respective species. Marine invertebrates are laden with associated bacteria (40% wet weight). Majority of the marine natural products show striking structural similarities to metabolites of microbial origin, suggesting that microorganisms are the real producers of the metabolites or mainly involved in their biosynthesis (Proksch *et al*., 2002). The supporting evidence for the involvement of natural product synthesis has been complied for the Didemnidae family for example *Lissoclinum patella* (ascidian) and cyanobactetial symbiont *Procholoron didemni*, Sponge *Dysidea herbacea* and their cyanobacterial symbiont *Oscillatoria spongiliae* and bryozoan *Bugula neritina*-symbiotic bacteria *Candidatus endobugula* association. Thus, the alternative strategy targeting the microorganisms associated with marine invertebrates and seaweeds for the screening of pharmacologically active natural products may prove to be an effective approach to depend whole organism itself which deals with biodiversity associated problem. Antibacterial activity of marine bacteria is a well known phenomenon and has been demonstrated in a number of studies (Uzair *et al*., 2006; Dash *et al*., 2009). However their ecological role and degrees of adaptation to the marine environment is largely unknown (Bush, 2004). A study of the epiphytic bacteria were isolated from marine algal surfaces indicated that the incidence of antibiotic producing colonies from this habitat was 20% whereas that from the seawater was only few percent (Lemos *et al*., 1985). Jensen *et al*. (2007) reported the associations between secondary metabolite production and phylogenetically distinct but closely related marine actinomycetes belonging to the genus *Salanispora* whereas Fang *et al*. (2009) screened PKS I gene from the epiphytic bacteria of *Porphyra* spp. based on the antibacterial analysis. Extracts of the Egyptian marine actinomycete, *Nocardia* sp. yield four structurally related bioactive compounds chrysophanol 8-methyl ether, asphodelin; 4,7'-bichrysophanol and justicidin B, in addition to a novel bioactive compound ayamycin; 1,1-dichloro-4-ethyl-5-(4-nitro-phenyl)-hexan-2-one (El-Gendy *et al*., 2008). The present study was made to carryout to evaluate the pharmacological activities such as the anti inflammatory, antinociceptive and central nervous system depressant activities of four promising bacterial strains BR1 isolated from barnacle *Balanus amphitrite*, PC4 from the ascidian *Polyclinum constellatum*, EM13 and EM14 from *Enteromorpha compressa*. These strains were selected from our previous study (Vijayalakshmi *et al*., 2008) based on their broad spectral antibacterial activity.

MATERIALS AND METHODS

Isolation of Marine Bacteria

Seaweeds were collected from Tuticorin (Gulf of Mannar, South East Coast of India) study was conducted from 2007 to 2008, Mahabalipuram (lat. 12°37'N and long. 80°14'E) and Kovalam coast (lat 12°47' 23.41"N and long. 80° 14' 52.72E) South coast of India) and that of ascidians from Tuticorin coast (lat. 8°45' N and long. 78° 13' E) South East coast of India. The study was carried out at Centre for Research and Development, PRIST University, Thanjavur, Tamil Nadu, India. The associated bacterial strains were selected based on their colony morphology.

Isolation of Seaweed Associated Bacteria

The seaweed species, *Chetomorpha linoids*, *Ulva lactuca*, *Enteromorpha compressa*, were collected for isolation of epiphytic bacteria. Samples were placed in sterile plastic bags underwater and brought to the lab for immediate processing. The seaweeds were rinsed with sterile seawater prior to analysis to remove loosely attached bacteria (Lemos *et al*., 1985). For isolation of ectosymbionts the

seaweed approximately 1 cm^2 surface were swabbed by sterile cotton swab and then placed in 2 mL sterile seawater and vortexed. Each solution was diluted 10 times for serial dilution. Then 0.1 mL aliquots were plated on Zobell marine agar (Himedia, Mumbai, India) and B_1 medium (2.5 g pep one, 1.5 g yeast extract, 1.5 mL glycerol, 17 g agar, 750 mL filtered seawater, 250 mL deionized water). This procedure was repeated in duplicate for each species by swabbing separate surface areas of seaweeds (Wahl *et al*., 1995; Chelossi *et al*., 2004).

For isolation of endo-symbionts the seaweed sample was homogenized with sterile seawater and then the bacteria were isolated in 3 different types of medium as mentioned in the above by following the pour plate technique. Triplicates were maintained and incubated at 27°C for 7 days. The individual bacterial strain was isolated by repeated streaking.

Isolation of Epibiotic Bacteria from Ascidians

The epibiotic bacteria were isolated from the four species of ascidians, *Polyclinum constellatum*, *Phallusia nigra*, *Didemnum psammathodes*. These four species of ascidians were collected from Hare Island of Tuticorin, Gulf of Mannar, South East Coast of India. The ascidians were collected separately in sterile polythene bags and aseptically brought to the laboratory. A sterile plastic film with a 0.5 cm^2 hole was placed on the animal surface and the area within the surface was swabbed with a sterile cotton swab and then this swab was placed in a tube containing 4 mL sterile seawater. This tube was vigorously vortex mixed yielding dilution 1. Dilution 1 was serially diluted with sterile seawater yielding dilutions 2-4. One milliliter of dilution were inoculated onto B_1 medium agar plates. The agar plates were incubated at room temperature for 7 days (Wahl, 1995). Isolation of barnacle associated bacteria. The Barnacle *Balanus amphitrite* were taken from the different rocks present inside the Port of Tuticorin and Mahabhalipuram coastal waters. Then 1 mg of organism with hard cement covering were taken and homogenized by mortar and pestle with sterile seawater and bacterial strains were isolated by the pour plate technique.

Preparation of Extracts

Isolated bacterial colonies were inoculated into separate conical flask containing seawater broth. Then they were kept in shake culture incubator for 7 days. After that they were extracted with equal volume of ethyl acetate and filtered through 0.22 μm filter and concentrated by rotary vaccum evaporator and then freeze dried to yield powder form of the bacterial extract. These crude extracts powder will be used for the pharmacological assays.

Pharmacological Procedures

Animals

Wistar albino rats of either sex/swiss albino mice were obtained from Animal facility of ArulmiguKalasalingam College of Pharmacy and Pharmacology Research Lab, Anand Nagar, Srivilliputhur, Tamil nadu, India. The animals were maintained in colony cages at 25±2°C in 10:14 light and dark. The animals were fed with standard balanced feed and water was applied *ad libitum*. A minimum of six animals group. All the animals were acclimatized to the laboratory conditions prior to experimentation. Tween 80 is used as a vehicle.

Acute Toxicity Studies

Acute toxicity studies were carried out by following acute toxic class method as per OECD guidelines 425 (OECD, 2002). Acute toxicity for various plant extracts was carried out using groups of three Swiss albino mice by administrating a dose 1000 mg kg^{-1} in saline solution while the control group received only the vehicle. These were observed mortality and behavioral changes during 48 h. Based on this observation 200 mg kg^{-1} i.p. were used for acute treatment in the following experiments.

Test Samples for Bioassays

The crude lyophilized marine bacterial extract suspended into saline water and used for bioassays. The control group received the same experimental handling as those of the test groups except the drug treatment was volume of dosing vehicle. Indomethacin (10 mg kg^{-1}), pentazocine (5 mg kg^{-1}), pentobarbitone sodium (40 mg kg^{-1}) and Diazepam (4 mg kg^{-1}) were used as a reference drugs.

Anti Inflammatory Activity-Induced Hind Paw Edema Model

Carragennan induced hind paw edema model was used for determination of anti inflammatory activity (Yesilada and Küpeli, 2007). The animals were fasted 18 h prior to the experiment. The extract was administrated orally (200 mg kg^{-1}). The paw thickness of the rats c was measured plethysmographically (LETICA-7500). Before administering carragenan (V_0) and after 1, 2, 3 and 4 h. The amount of paw swelling was determined for each rat and the difference between (V_T) (1, 2, 3 and 4 h) and (V_0) was taken as the edema value. Indomethacin (10 mg kg^{-1}) was used as reference drug. The percentage of inhibition was calculated according to the following formula:

$$\text{Percent inhibition} = \frac{(V_T - V_0)\text{control} - (V - V_0)\text{treated group}}{(V_T - V)} \times 100$$

Antinociceptive Activity-Hot Plate Reaction Method

The mice were selected 24 h before the study on the basis of their reactivity in the test using cut off time 10 sec. Selected mice were giving the extract (200 mg kg^{-1}), pentazocine (Positive control, 95 mg kg^{-1}) and control mice with vehicle saline (10 mg kg^{-1}) i.p. After 30 min each mouse was placed on the heated surface of the hot plate maintained at 55± 0.5°C and the reaction time was measured when the animal jumped off the plate or licked its paw. The cut off time of 20 sec was imposed to avoid tissue damage (Eddy and Leimbach, 1953; Toma, 2003).

Central Nervous System Depressant Activity (CNS)

For the evaluation of locomotor activity, healthy and adult male swiss mice weighing 20-30 g, fasted for 24 h prior to experiment. The basal activity score for all the animals were recorded. The control group was given only 10% v/v Tween 80 (vehicle) Suspension. The standard drug diazepam at a dosage 4 mg kg^{-1} was administered to one group of animals intraperitoneally. Scores were recorded after 30 min for all animals and percentage change of activity was calculated by the following formula:

$$\text{Change in motor activity} = (A-B)/A \times 100$$

Where:
A: Basal score
B: Score after treatment

For the evaluation of Pentabarbitone sodium induced sleeping time, after 30 min of control as well as extract, injected groups were administrated intraperitoneally at a dose of 40 mg kg^{-1}. The time of administration of the test compounds and the phenobarbitone sodium and the time of loss and gain of righting recorded in all the groups of test animals and the percentage effect on phenobarbitone narcosis by test compounds was calculated using the formula given below, considering righting reflex in control as 100%.

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

RESULTS

The ethyl acetate extracts of four marine bacteria BR1, PC4, EM13 and EM14 isolated from barnacle *Balanus amphitrite*, ascidian *Polyclinum constellatum* and seaweed *Enteromorpha compressa* respectively were investigated for anti inflammatory activity using carrageenan-induced hind paw

Table 1: Effect of marine bacterial extracts isolated from *Balanus amphitrite* (Barnacle), *Polyclinum constellatum* (ascidian) and *Enteromorpha compressa* (seaweed) on carrageenan-induced hind paw edema in mice

		Swelling thickness ($\times 10^{-2}$ mm)±SEM (% inhibition)			
	Dose	60	120	180	240
Drug	(mg kg^{-1})	(min)			
Control (Saline)	-	38.61±4.56	80.86±2.92	96.76±0.2	110.82±5.02
Indomethacin	10	12.25±2.92* (68.27)	20.28±3.8 (74)	26.27±3.22 (72.8)	3.60±3.42* (69.68)
BR1-(EtOAc)	200	3.26±5.5 (13.85)	2.38±4.20 (22.85)	91.80±3.20 (5.56)	103.86±5.66 (6.8)
PC4-(EtOAc)	200	1.38±3.48* (54.98)	51.68±3.80* (36.09)	83.32±5.12 (13.89)	91.21±3.98* (17.69)
EM13 (EtOAc)	250	1.62±2.98* (59.54)	45.62±2.92* (43.58)	52.20±3.62 (45)	88.21±2.86* (20.40)
EM14-(EtOAc)	200	18.72±3.38* (5.51)	56.26±2.82* (30.42)	51.26±2.80* (47.02)	57.42±3.68* (48.18)

SEM: Mean standard error. *p<0.05 as compared to control group significant from control group. EtOAc: Ethyl acetate, BR1: Bacterial strain isolated from Barnacle Balanus amphitrite, PC4: Bacterial strain isolated from the ascidian *Polyclinum constellatum*, EM13 and EM14: Bacterial strain isolated from the seaweed *Enteromorpha compressa*

Table 2: Effect of marine bacterial extracts isolated from *Balanus amphitrite* (Barnacle), *Polyclinum constellatum* (ascidian) and *Enteromorpha compressa* (seaweed) on the hotplate test in mice

		Reaction time in seconds at time (min)				
Drug	Dose (mg kg^{-1})	0	30	60	120	180
Control	--	8.65±1.62	8.79±1.08	8.10±0.68	13.20±1.21	10.82±1.36
Pentazocine	5	9.32±1.06	10.84±1.37	15.32±2.09*	18.44±0.52**	13.88±0.94
BR1	200	9.25±1.52	7.22±0.64	8.98±0.73	14.22±1.20	11.86±1.20
PC4	200	8.49±1.26	8.26±1.22	10.26±1.72	16.20±1.06	13.76±2.08
EM13	200	8.42±1.72	8.16±1.21	11.26±1.81	13.90±1.06	12.82±2.06
EM14	200	8.51±0.80	10.47±1.05	10.12±0.64	16.89±1.66	16.26±1.65*

SEM: Mean standard error. *p<0.05, *p<0.01 as compared to control group significant from control group. EtOAc: Ethyl acetate, BR1: Bacterial strain isolated from Barnacle *Balanus amphitrite*, PC4: Bacterial strain isolated from the ascidian, EM13 and EM14: Bacterial strain isolated from the seaweed *Enteromorpha compressa*

Table 3: Effect of marine bacterial extracts isolated from *Balanus amphitrite* (Barnacle), *Polyclinum constellatum* (ascidian) and *Enteromorpha compressa* (seaweed) on Locomotor activity

		Locomotor activity scores in minute		
Compound	Dose (mg kg^{-1} i.p. route)	Before treatment	After treatment	Percentage change in activity
Control	0.5 mL	32.56	32.50	0.18
Diazepam	4	39.22	10.28	73.78
BR1	200	35.20	26.32	25.23
PC4	200	36.88	30.88	16.27
EM13	200	38.66	22.41	42.03
EM14	200	36.52	28.20	22.78

Average 6 determinations

Table 4: Effect of marine bacterial extracts isolated from *Balanus amphitrite* (Barnacle), *Polyclinum constellatum* (ascidian) and *Enteromorpha comprissa* (seaweed) on Phenobarbitone sodium (40 mg kg^{-1}) induced sleeping time

Compound	Dose (mg kg^{-1} i.p. route)	On set of action min	Sleeping time min	Percent effect
Control	0.5 mL	14.40±2.06	33.40±3.09	100.00
BR1	200	12.30±2.01	93.44±1.42	279.38
PC4	200	13.31±1.99	70.10±2.12	209.88
EM13	200	11.25±2.05	95.25±1.92	285.18
EM14	200	9.32±1.34	70.50±2.01	211.08

SEM: Mean standard error. *p<0.05, *p<0.01 as compared to control group significant from control group. EtOAc: Ethyl acetate, BR1: Bacterial strain isolated from Barnacle *Balanus amphitrite*, PC4: Bacterial strain isolated from the ascidian *Polyclinum constellatum*, EM13 and EM14: Bacterial strain isolated from the seaweed *Enteromorpha compressa*

edema, antinociceptive activity using hot plate method and central nervous system depressant by using phenobarbitone induced sleeping time at 200 mg kg^{-1} dose of body weight. The experimental results were shown in Table 1-4.

The ethyl acetate extracts of the bacterial strains EM13 and EM14 provided remarkable anti inflammatory activity , the percentage of inhibition ranges from 20.4-59.5% ($p<0.05$) and the BR1 strain exhibited lesser anti-inflammatory activity than other 3 extracts at the dose of 200 mg kg^{-1}. However, the marine bacteria PC4 (20.4-59.5%) which showed higher anti inflammatory activity but lesser than EM13 and EM14. The results were statistically significant when compared to the standard reference compound (Indomethacin) (Table 1).

The extract of EM14 (at the dose 200 mg kg^{-1}) found to be greater than standard drug at 3 h which showed highest antinociceptive activity among the four extracts at the dose of 200 mg kg^{-1}. The antinociceptive activity of all four marine bacterial extracts was significant ($p<0.05$) compared to the standard drug pentazocine ($p<0.05$, $p<0.01$). The activity was effective in later phases and lasted until the end of the experiment. The activity started diminished after 3 h (Table 2).

Diazepam was used as standard drug. Of all four bacterial extracts the ethyl acetate extract of the EM13 exhibited higher percentage (42.03%) of reduction in locomotor activity. The standard drug diazepam treated animal 73.78% reduction in locomotor activity (Table 3).

In Table 4 the percent effect of phenobarbitone sodium induced sleeping time of all 4 bacterial extracts were shown. The ethyl acetate extract of BR1 showed higher percent of induced sleeping time next to EM13. The experiment is significant as compared to the control group ($p<0.05$, $p<0.01$).

DISCUSSION

The overall objective of the current study was to evaluate the anti inflammatory , analgesic and CNS depressant activities of four promising bacterial extracts BR1, PC4, EM13 and EM14. All four strains exhibited anti inflammatory activity in addition to broad spectral antibacterial activity (four strains were selected based on promising antibacterial activity). This observation is coincided with Bobzin and Faulkner (1991), who have isolated Chelonin A isolated from the sponge *Chelonaplysilla* sp. found in a marine lake Palau exhibited both anti inflammatory and antibacterial activity. Both anti inflammatory and analgesic activities probably mediated through a common mechanism. All the extracts significantly reduced the formation of paw edema induced by Carragennan and increased reaction latency to thermal pain in mice.

The Carragennan-induced inflammation induced by the activation of the kinin system, the accumulation of neutrophils and the release of several mediators such as prostanoids and cytokines (Akkol *et al*., 2008). Carragennan induced hind paw edema in rat includes three distinct phases. The release of histamine and serotonin in the first phase (0-120 min). Kinins in the second phase (180 min) and prostaglandin in the third phase (240 min) (Olumayokun *et al*., 1999). So the present study was made for 4 h of observation. In order to study the effect of the extracts on the histamine and serotonin synthesis, the observation was made by inducing the inflammation by histamine and serotonin (data not shown). It was observed that these four marine bacterial of inhibiting edema induced by histamine and serotonin.

The hotplate test is thought to involve the spinal reflex and is regarded as one of the suitable models for determining the involvement of central antinociceptive mechanism (Hosseinzadeh and Younesi, 2002). The exposure of animal to thermal stimuli in the hot plate test will lead to the development of a non-inflammatory, acute nociceptive response and the ability of the extract to inhibit the thermal induced nociceptive indirectly indicates its ability to inhibit non inflammatory pain. The fact that extract inhibits both types of tests suggested that it is possessed a centrally mediated activity like morphine. The study indicated the bacterial extracts have both peripheral and central analgesic properties. The centrally acting protective extracts were corroborated by the first phase of hot plate test.

The locomotor activity of the animal was reduced by all the extracts of the marine bacteria. In control the activity was negligible. The prolongation of phenobarbitone sleeping time and suppression of exploratory activity confirm the CNS depressant activity of the extracts. The CNS depressant activity is prolonged phenobarbitone induced sleeping time. The four potential marine bacterial extracts not only caused the reduction in motor activity but also potentiated the activity of phenobarbitone sodium induced sleeping time.

Phenobarbitone is one type of hypnotic agent, when give at appropriate dose, induces sedation or hypnosis in animals by potentiating the GABA mediated post synaptic inhibition through an allosteric modification of GABA receptors (Goodman and Gilman's, 2001). So, the possible mechanism of CNS depressant activity by these extracts may be attributed to the enhancement of GABA in brain. The results of the present study are promising, found that four potential bacterial strains exhibit anti inflammatory, antinociceptive and CNS depressant activities. Of these, EM13 and EM14 are found to produce highly active compounds.

CONCLUSION

It can be concluded that the results of the present study have shown that all the four extracts (EM13, EM14, PC4 and BR1) exhibited anti-inflammatory, antinociceptive and CNS depressant activities. This implies that the presence of one or more pharmacologically active compounds in these promising epibacterial strains. Though PC4 and BR1strains displayed anti inflammatory, antinociceptive and CNS depressant activities EM13 and EM14 are the potential strains. The present investigation highlights the importance of bacteria associated with sessile marine organisms and this bacteria is one of the richest source for the discovery of novel bioactive principles. Further isolation of active compounds from these bacterial extracts and the phylogenetic identification based on 16 s rRNA of all the 4 promising strains are being carried out progress.

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