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Pharmacological Activities of Marine Bacteria *Bacillus megaterium* and *Pseudomonas aeruginosa*

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Abstract: Background: Marine bacteria *Bacillus megaterium* associated with cone snail and *Pseudomonas aeruginosa* from tubeworm were cultured and the chloroform extract of the culture supernatants were screened for Central Nervous System (CNS) depressant, anti-inflammatory, analgesic and antipyretic activities. **Results**: The locomotor activity in rats was greatly reduced by both extracts and the activity was dose dependent. The *B. megaterium* strain extract showed higher depressant activity. Though the groups treated with test extracts of *B. megaterium* and *P. aeruginosa* showed increase in volume of paw edema, anti-inflammatory activity was noticed up to 30 min except in *P. aeruginosa* 200. *B. megaterium* extract at 200 mg kg^{-1} was potent as it showed higher activity even at 60 min than the standard drug. In analgesic activity screening, the reaction time increased with increasing extract concentration and time. Both extracts produced significant antipyretic effect in a dose dependant manner. The CNS depressant and analgesic activities were pronounced in both extracts. The chloroform extract of *B. megaterium* and *P. aeruginosa* showed significant pharmacological activities. The chloroform extract of both strains showed potent to Central Nervous System depressant, anti-inflammatory, analgesic and antipyretic activities.

Key words: Analgesic, anti-inflammatory, antipyretic, *Bacillus megaterium*, central nervous system depressant, marine bacterial metabolites, *Pseudomonas aeruginosa*

INTRODUCTION

The world's oceans, which cover almost 70% of the earth's surface and over 90% of volume of its crust, encompass a diverse array of fauna and flora, many of which have no terrestrial counterparts. The ocean life is represented by 34 of the 36 phyla in contrast to the 17 phyla of the terrestrial environment (Faulkner, 2000). Oceanic organisms, which constitute a major share of the earth's biological wealth, possess unique structures, metabolic pathways and provide structurally varied group of pharmacologically dynamic compounds to mankind. The bacteria, fungi, algae, sponges, soft corals, tunicates, molluscs and bryozoans are the most interesting organisms of pharmacological significance inhabiting the complex ecosystems of the marine environment (Faulkner, 2002).

A wide range of useful drugs including antibiotics, analgesics, anti-inflammatory, anticoagulants, CNS depressants, antipyretic agents etc. have been isolated from marine organisms. Currently, only few marine derived products are in the market and several of them are in clinical trials (Thakur *et al*., 2005). Various marine toxins like tetradotoxin and saxitoxin are not only important pharmacological tools, but can be used in terminal cases of cancer for relief of pain (Munro *et al*., 1987). Compounds isolated from marine organisms such as manoalide, a sesterterpene from the sponge *Luffariella variabilis* (Potts *et al*., 1992), sphingosine derivative and the cembrenoid diterpene from soft corals of *Simularia crassaa* and *Lobophytum* species (Loukaci *et al*., 2000) possess anti-inflammatory activity.

Microbes always played a vital role in the development of several drugs. Natural products from microbes, especially the associated organisms, remain one of the most important sources of lead compounds for the pharmaceutical industry and some have been synthesized and others serve as leads in the synthesis of bioactive analogs (Radwana and El-Sherbiny, 2007). Marine microbes are unique producers of bioactive metabolites than their terrestrial counterparts. But, the non-culturability of the majority of the microbes

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possesses great challenge. In many instances, the compounds isolated from marine invertebrates have been linked to their associated microbes. Hence, the present study was initiated to screen the associated microbes of the marine organisms for potential activity of biomedical importance. The chloroform extracts of *Bacillus megaterium* isolated from the marine snail *Conus virgo* and *Pseudomonas aeruginosa* from *Hydroides* sp. were evaluated for the pharmacological properties like Central Nervous System (CNS) depressant, anti-inflammatory, analgesic and antipyretic activities.

MATERIALS AND METHODS

Extraction: The producer strains *Bacillus megaterium* isolated from cone snail *Conus virgo* and *Pseudomonas aeruginosa* from the tubeworm *Hydroides* sp. were broth cultured in 100 mL Zobell marine broth individually for five days at 290 rpm at room temperature. The culture broth was then centrifuged at 5000 rpm for 10-15 min and then the supernatant was extracted employing liquid-liquid extraction (Gailliot, 1998). Chloroform was used for extraction based on activity profile against bacterial pathogens. Equal volume of chloroform was added to the broth and stirred for 5-10 min, using a magnetic stirrer. The two phases were then separated in a separating funnel and the solvent phase was concentrated by evaporation. The concentrate (crude extract) was used for the assessment of pharmacological activities.

Experimental animals: Adult albino rats of either sex weighing between 100-200 g were used. The animals were housed under standard environmental conditions with an alternating 12 h light-dark cycle and relative humidity of 60±5% in the Department of Pharmacology, S.B. College of Pharmacy, Sivakasi and were given uniform pelleted diet and water *ad libitum*. The active producer strains *B. megaterium* and *P. aeruginosa* were mentioned with their codes throughout the text. Prior approval of Institutional Ethic Committee was obtained for the experiments.

Central nervous system (CNS) depressant activity: Spontaneous locomotor activity and rearing were measured using a computerized locomotion detection system (actophotometer) equipped with photosenser (Asakura *et al*., 1993). Thirty minutes after the oral administration of test compound, an albino rat was individually placed in a transparent cage (25×48×18 cm^3) and locomotor activity and rearing were recorded for 10 min. To evaluate the interaction between test compounds and diazepam, animals were divided into six groups. Group I served as an untreated control and group II was orally treated with standard diazepam (3 mg kg^{-1}, p.o.). Group III and IV were treated with test compounds (*P. aeruginosa*) at concentrations of 100 and 200 mg kg^{-1} and group V and VI were treated with test compounds (*B. megaterium*) at concentrations of 100 and 200 mg kg^{-1}. Tween 80 was used as suspension medium. The locomotor activity was observed after 30 min of the extract administration for 10 min and the percentage change in the activity was calculated.

Anti-inflammatory activity: Carrageenan-induced rat paw edema: Albino rats were divided into six groups of four animals each. The control group was given saline (1 mL kg^{-1}) orally. Anti-inflammatory activity was evaluated by injecting carrageenan (Sigma, 0.05 mL of 1% w/v) subcutaneously into the sub-plantar region of the right hind paw and the induced paw edema was measured. Saline (1 mL kg^{-1}) given to Group I was used as carrageenan treated control and the standard drug Diclofenac sodium (10 mg kg^{-1}) was administered to Group II rats. One hour prior to carrageenan injection, Group III and IV were treated with test extract of *P. aeruginosa* bacteria and Group V and VI were treated with of *B. megaterium* bacterial extract (in saline) at a dose level of 100 and 200 mg kg^{-1} p.o. All the doses were administered orally. The thickness of right paw was measured before and after carrageenan injection at time intervals of 0, 15, 30 and 120 min, respectively and the percentage increase of paw edema thickness was calculated (Duwiejua *et al*., 1994).

Analgesic activity: Tail immersion method: Adult albino rats were screened for sensitivity by placing the tip of the tail (last 1-2 cm) gently in warm water maintained at 55±2°C. Any rats flicking the tail within 5 sec were selected for the study. The selected rats were divided into six groups of four animals each. The Group I (control group) received normal saline whereas the Group II (standard reference group) was treated with (4 mg kg^{-1}) p.o. of Pentazocine. Group III and IV received *B. megaterium* bacterial chloroform extracts of 100 mg and 200 mg kg^{-1}. p.o., respectively. Group V and VI have received BM bacterial chloroform extracts at 100 mg and 200 mg kg^{-1}. p.o., respectively. After drug treatment, the basal reaction time of all groups of animals was noted at different time intervals like 15, 30, 60 and 120 min (Kulkarni, 1999).

Antipyretic activity: Antipyretic activity was carried out by using Digital Telethermometer (TNCO). Male albino rats having the rectal temperature 35-38°C were selected.

The animals were divided into six groups of four animals each. Pyrexia was induced in albino rats by injecting 20% (M/V) aqueous suspension of Brewer's yeast in the nape subcutaneously (Rajasekaran *et al*., 1999; Asha and Pushpangadan, 1999). After 18 h the animals developing 0.5°C rise in the rectal temperature were selected for further studies. The Group I (control group) received normal saline whereas the Group II (standard reference group) was treated with (45 mg kg^{-1}) p.o. of Paracetamol. Group III and IV were treated with *P. aeruginosa* bacterial chloroform extracts at 100 mg and 200 mg kg^{-1}, p.o., respectively. Group V and VI received *B. megaterium* bacterial chloroform extracts at 100 mg and 200 mg kg^{-1}, p.o. The rectal temperature was recorded at 1, 2 and 3 h after administration of the test drug extracts.

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

RESULTS AND DISCUSSION

The world's oceans has been viewed as the most important and productive source of biomedical compounds by the pharmaceutical industry. The marine drug discovery has a bright future as has been visualized by the current research activities (Fenical, 1997). The marine microbes have been the source of potential pharmacological activities. The alkaloid oxepinamides isolated from marine fungus *Acremonium* sp. (Belofsky *et al*., 2000) and the scytonemin pigment, isolated from marine cyanobacteria (Proteau *et al*., 1993) are the typical examples of marine microbial compounds showing anti-inflammatory activity.

CNS depressant activity: The CNS depressant activity of chloroform extracts of *B. megaterium* and that of *P. aeruginosa* were lower when compared to the standard drug Diazepam (5 mg kg^{-1}). The results were comparable to the CNS depressant activity of an ascidian *Distapila nathensis* (Rajasekaran *et al*., 2003) at 100 mg kg^{-1} and that of scallop *Minnivola pyxidata* (Jaya Seeli, 2004) which induced drowsiness, but not sleep in the test animals indicating CNS depressant activity. In our study, the locomotor activity of the rats was greatly reduced by the extracts and the activity was dose dependent with higher dose showing higher depressant activity (Table 1). The standard drug Diazepam (5 mg kg^{-1} p.o.) treated rats showed a reduction of 96.6±0.4% in locomotor activity. Comparatively, the bacterial extracts showed less depressant activity. The chloroform extract of *B. megaterium* exhibited a higher reduction (92.3±0.5%) in the locomotor activity at 200 mg kg^{-1} p.o. than that of 90.8±1.1% observed for *P. aeruginosa* at the same concentration. The *B. megaterium* strain extract showed higher depressant activity.

Anti-inflammatory activity: The Carrageenanan induced paw edema method is generally used to evaluate the effect of Non-steroidal Anti-inflammatory Drugs (NSAIDs) (Phadke and Anderson, 1988). In the present study, the 100 mg kg^{-1} *P. aeruginosa* extract showed marked anti-inflammatory activity during the first 15 min observation. It also showed comparatively better activity next to standard drug. The activity of the extracts was higher during first 30 min than the standard drug. After 30 min the lowering trend was noticed with time. Interestingly, lower concentrations of extracts showed higher inhibitory activity. The observation was substantiated by isolation of scytonemin, an extracellular sheath pigment from cyanobacterium *Stigonema* sp., which was reported to possess anti-inflammatory activity (Stevenson *et al*., 2002). The fact that the associated microbes were the actual producers of the active metabolites was substantiated by the pseudopterosins, tricyclic diterpene glycosides isolated from the Caribbean sea whip *Pseudopterogorgia elisabethae*, which was shown to possess anti-inflammatory and analgesic activities (Look *et al*., 1986), was reported to originate from the dinoflagellate symbiont *Sympoidinium* sp., localized within the tissues of the sea whip (Mydlarz *et al*., 2003). The increase in volume of paw thickness for control was 0.4 mm. The *B. megaterium* chloroform extracts also showed similar increase in volume at concentrations of 100 and 200 mg kg^{-1} p.o. (Table 2).

The standard drug Diclofenac sodium had some effect and showed anti-inflammatory activity with reduced paw volume of 0.32 mm. Though the groups treated with test extracts of *B. megaterium* and *P. aeruginosa* showed increase in volume of paw edema, effective anti-inflammatory activity was observed at 15 min ($p<0.05$) and it extended up to 30 min. At 60 min. the *B. megaterium* 200 mg kg^{-1} extract showed higher activity ($p<0.05$) than the standard drug. At 120 min all the extracts showed lower activity than the standard drug.

The anti-inflammatory effect of the *B. megaterium* and *P. aeruginosa* extracts in the present study was comparatively less than the reported moderate anti-inflammatory effect of the methanolic extracts of *Cypraea errones* and *C. Arabica* (Kumar, 2003) against Carrageenan-induced inflammation at a dose of 10 mg kg^{-1}. Also, new sphingosine derivative and cembrenoid diterpene obtained from marine soft corals

Table 1: Effect of *B. megaterium* and *P. aeruginosa* chloroform extracts on locomotor activity (CNS depressant activity)

		Mean of locomotor activity scores in 10 min			
Treatment	Dose (mg kg^{-1})	Before treatment	After treatment	Change in locomotor activity (%)	Nature of action
Control	-	292	292	70.0	-
Diazepam	5	324	11*	96.6	Depressant
PA	100	218	24*	88.9	Depressant
	200	250	23*	90.8	Depressant
BM	100	183	14*	90.8	Depressant
	200	184	17*	92.3	Depressant

n = 4, *significant values at p<0.05

Table 2: Anti-inflammatory activity of *B. megaterium* and *P. aeruginosa*extracts against carrageenan induced paw edema in albino rats

	Paw edema (mm±SD)					
Dose (mg kg^{-1})	0 min	15 min	30 min	60 min	120 min	Increasing volume of paw edema (mm)
Control	-	0.20±0.10	0.35±0.08	0.43±0.10	0.60±0.10	0.40
Diclofenac sodium	-	0.15±0.06	0.25±0.10	0.37±0.82	0.47±0.07*	0.32
PA100	-	0.07±0.00*	0.20±0.14	0.40±0.10	0.45±0.09*	0.38
PA 200	-	0.10±0.00*	0.42±0.05	0.52±0.05	0.55±0.05	0.45
BM 100	-	0.10±0.00*	0.17±0.00*	0.40±0.00	0.50±0.00*	0.40
BM 200	-	0.10±0.00*	0.22±0.05*	0.31±0.06*	0.50±0.12	0.40

n = 4, *significant values at p<0.05

Table 3: Analgesic activity of *B. megaterium* and *P. aeruginosa* extracts by tail flick method

		Reaction time (sec) after drug administration (±SD)			
Treatment	Dose (mg kg^{-1})	15 min	30 min	60 min	120 min
Control	-	2.00±0.58	2.50±0.58	2.50±0.58	2.75±0.58
Pentazocine	4	6.25±0.50	7.75±0.50	8.50±0.50	11.0±0.82*
PA	100	5.50±0.58	7.25±0.58	8.25±0.58	9.50±0.58*
	200	5.75±1.00	8.00±1.41	8.75±1.00	9.75±1.29*
BM	100	5.25±0.96	7.00±0.96	8.25±0.96	9.25±0.58*
	200	6.25±0.50	7.50±0.82	8.5±0.500	10.50±0.82*

n = 4, *values are significant of p<0.05

Sinularia crassa and *Lobophytum* species have been reported to exhibit anti-inflammatory activity at a dose of 5 mg and 10 mg kg^{-1} (Radhika *et al*., 2005). The results were also low when compared to the inhibition of paw edema to the extent of 60% from *Hypnea musciformis* (Solimabi, 1980). The low level activity could be due to the use of crude extracts and the possibility of other constituents interfering with the inhibition at higher concentration was not ruled out.

Analgesic activity: The administration of standard drug Pentazocine (4 mg kg^{-1} p.o.), resulted in higher reaction time during the experiment. At 15 min, *B. megaterium* 200 mg kg^{-1} extract showed equal reaction time with that of standard drug (Table 3). At 30 and 60 min, the *P. aeruginosa* 200 mg kg^{-1} extract showed higher reaction time. At 120 min, all the extracts showed lower reaction time than that of standard drug. The reaction time increased with increasing extract concentration and time (p<0.05).

The analgesic activity of the chloroform extracts of *B. megaterium* and that of *P. aeruginosa* were lower when compared to the standard drug Pentazocine. It may be due to the fact that the standard drug is in a pure form and that of extracts were in a crude form. But, the extract of *P. aeruginosa* showed higher activity than the standard drug at 30 and 60 min at 200 mg kg^{-1} concentration. In general, the extracts comparatively showed good time and dose dependent analgesic activity and the results were comparable to pseudopterosins isolated from the gorgonian *Pseudopterogorgia elisabethae* (Look *et al*., 1986), which exhibited anti-inflammatory and analgesic activities and that of acetone and methanol extracts of scallop *Minnivola pyxidata* (Jaya Seeli, 2004), which exhibited analgesic activity by inducing the rats to tolerate temperature up to 8 to 8.5 sec.

Antipyretic activity: Yeast induced pyrexia (Mukherjee *et al*., 1996) is a classical method of testing antipyretic activity. Using this method several investigators recorded pyrexia two or four hours after injection and then they administered the antipyretic drugs to be studied. In the present study, the temperature reduction of 2 and 2.3°C and 1.9 and 2.2°C for *B. megaterium* and *P. aeruginosa* extracts at the concentrations of 100 and 200 mg kg^{-1}, respectively indicated the antipyretic activity of the extracts, though lower than the standard drug. It was comparable to the

Table 4: Antipyretic activity of *B. megaterium* and *P. aeruginosa* extracts against Brewer's yeast induced pyrexia in albino rats

(mg kg^{-1})	Fasting rectal Temp. (°C)	Initial pyrexia	1st h	2nd h	3rd h	Reduction of dose Temp. (°C)
Control	36.4±0.39	38.6±0.91	38.9±0.88	36.0±0.65	39.6±0.59	-
Paracetamol 500	37.8±0.39	38.5±0.33	35.2±0.66	34.5±0.60	34.3±0.55*	4.2
PA 100	36.1±0.31	37.2±0.16	36.1±0.42	36.1±0.34	35.3±0.21*	1.9
PA 200	35.7±0.39	37.5±0.33	36.4±0.66	36.4±0.60	35.3±0.55*	2.2
BM 100	35.8±0.10	37.2±0.30	36.2±0.40	36.2±0.20	35.2±0.20*	2.0
BM 200	35.7±0.25	37.5±0.24	36.2±0.34	36.2±0.17	35.2±0.26*	2.3

n = 8, values are ±SD, *significant at $p<0.05$

temperature reduction of 1.8 and 1.9°C after three hours after administration of extract of *Cypraea errones* (10 mg kg^{-1}) and *Cypraea arabica* (10 mg kg^{-1}) (Kumar, 2003), respectively. The extracts of either concentration didn't show any substantial variation, though higher concentration showed higher reduction of temperature. The standard drug showed moderation after 1st h and the reduction in temperature was almost stabilized. But, extracts which showed moderate activity during the 1st and 2nd hour, showed higher activity during 3rd hour So, the action of the extracts may be slow in contrast to the standard drug. This may be attributed to the crude form of the extracts also.

Both extracts produced significant antipyretic effect in a dose dependant manner (Table 4). The *B. megaterium* extracts exhibited 2 and 2.3°C of reduction of temperature at the concentrations of 100 and 200 mg kg^{-1}, respectively. The *P. aeruginosa* extracts exhibited 1.9 and 2.2°C of reduction of temperature at the concentrations of 100 and 200 mg kg^{-1}, respectively. The decrease in temperature for both bacteria at all concentrations was significant ($p<0.05$).

CONCLUSION

The study indicated Central Nervous System (CNS) depressant, anti-inflammatory, analgesic and antipyretic activities of chloroform extracts of culture supernatants of marine cone snail and tube worm associated bacteria *B. megaterium* and *P. aeruginosa* strains. The CNS depressant and analgesic activities were pronounced in both strains and further exploration of which would certainly lead to isolation of potentially useful compounds of biomedical importance.

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