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Antimicrobial compounds with therapeutic potential from *Cerithidea cingulata* against human and fish pathogens

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Abstract

The use of antimicrobial drugs to control infectious diseases must be among the greatest achievements of medicine in the last century. Marine Biotechnology and Aquaculture has developed into a prime industry to tap the enormous turnover of bio-energy for the benefit of mankind. Aquaculture has been the world's fastest growing food production system for the past decade. The air-dried samples of Cerithidea cingulata were immersed separately in ethyl acetate, acetone, hexane, chloroform, diethyl ether, dichloromethane, acetonitrile, methanol, butanol and cold steeped for overnight at -18^{0}C. The antibacterial effects of the extracts were tested against human pathogens (Bacillus cereus, Staphylococcus aureus, Vibrio cholerae, Enterobacter aerogenes and Klebsiella pneumoniae) and fish pathogens (Proteus mirabilis, Aeromonas hydrophila, Serratia marcescens, Aeromonas formicans and Micrococcus sp). The extract obtained from ethyl acetate showed a higher degree of inhibition to the fish pathogens too, similar to that of human pathogens. Maximum zone of inhibition was exhibited against S.marcescens (3.5 mm) by the ethyl acetate extract. The wide spectrum antibacterial activity exhibited by the whole body extracts of C.cingulata indicates that it may possess biologically active metabolites.

Keywords: *Cerithidea cingulata,* antibacterial agents, human pathogens, fish pathogens, solvent extraction.

Introduction

Modern medicine is dependent on chemotherapeutic agents. The use of antimicrobial drugs to control infectious diseases must be among the greatest achievements of medicine in the last century [1]. After many clinically useful antibiotics like streptomycin, chloramphenicol, chlortetracycline, neomycin, oxytetracycline, erythromycin etc. were discovered, the most bacterial infections seemed to be conquered. However a decade after the spread of antibiotic therapy a number of species of *Staphylococcus, Mycobacterium* and Gram negative enteric bacteria had developed resistance to antibiotics [17]. Antibiotics must be used in a concentration low enough to avoid undesirable damage to the host, which, at this stage, is not possible with many of the currently used synthetic antibiotics. The highest level of antibiotic resistance was determined in bacteria inhabiting seafoods, compared to water and sediment isolates [2].

Aquaculture has been the world's fastest growing food production system for the past decade [15, 26]. In 1997, over 30% of food consumed by humans was provided by aquaculture [11]. In Asia, shrimp viral diseases caused farmers about 1 billion-dollar loss every year since 1994 [27]. The disease was initially controlled almost exclusively by the use of antimicrobial drugs. The increasing intensity of shrimp farming was inevitably paralleled by the increasing incidence of diseases in the farming systems. Pathogens such as *Vibrios* are

the members of the normal bacterial flora of shrimps and act as primary and secondary invaders of shrimps in the culture system [14].

The massive use of antimicrobials for disease control and growth promotion in animals increases the selective pressure exerted on the natural emergence of bacterial resistance [28]. Transferable drug resistance has also been demonstrated in sediments beneath fish farms receiving oxytetracyclin [24]. In the past 20 years, the pharmacological industry has been relatively successful in containing problems due to single resistance determinants. However the advent of multiple resistance mechanism has severely reduced the use of currently used antibiotics. There is a growing concern about the use and particularly the abuse of antimicrobial drugs not only in human medicine and agriculture, but also in aquaculture. Hence the need of the hour is a search for novel antibacterial compounds with therapeutic potential for which the pathogens may not have resistance [18]. The search for new antibiotics is a continuous process.

Approximately 2,500 new metabolites were reported from a variety of marine organisms during the decade from 1977-1987 [13]. Due to the diversity of marine organisms in various habitats, marine natural products encompass a wide variety of chemical classes including terpenes, shikimates, polyketides, acetogenins peptides and alkaloids of varying structures and multitude of compounds of mixed biosynthesis. The marine environment yielded prolific producers of terpenoids, especially diterpenes [10].

Among the marine organisms, mollusks are one of the most successful forms of animal life and they have conquered almost every habitat and exist in all the oceans (from shallow tidal pools to the deepest trenches). Many studies on bioactive compounds from mollusk exhibiting antibacterial, antitumour, antileukemic, and antiviral activities have been reported worldwide [12, 7, 8, 20]. The inter-tidal mollusk, *Cerithidea cingulata* is a common inhabitant in the mats of the algae *Enteromorpha*. So far this gastropod has been little utilized in the marine natural product chemistry and an attempt is made to evaluate the antibacterial properties of this organism.

The emergence of the resistant bacteria has created a major concern and an urgent need for new antibacterial agents [5]. Marine organisms have been a rich source of novel chemical compounds [9], which have boosted the development of marine natural products chemistry for about 3 decades. Screening for antimicrobial activity has proven particularly simple, cost efficient and effective [22, 7].

Materials and methods

This marine organism *C.cingulata* lives in inter-tidal area mainly between mid-tide levels to low-tide level and feeds on detritus matter. Live *C.cingulata* were collected during low tide period in the inter-tital area of Tuticorin coastal waters, Southeast coast of India. The samples were immediately brought to the laboratory and washed with tap water to remove contaminants. The shells were broken and soft bodies of the organisms were air-dried. Approximately 100 g of the air-dried sample was immersed separately in different solvents, ethyl acetate, acetone, hexane, chloroform, diethyl ether, dichloromethane, acetonitrile, methanol and butanol and they were cold steeped overnight at -18^0C. The extracts from each solvent were filtered twice using Whatman No.1 filter paper. The filtrates were dried and used for the experimental work.

The antibacterial effect of the extracts was tested against human pathogens (*Bacillus cereus, Staphylococcus aureus, Vibrio cholerae, Enterobacter aerogenes* and *Klebsiella pneumoniae*) and fish pathogens (*Proteus mirabilis, Aeromonas hydrophila, Serratia*

marcescens, Aeromonas formicans and *Micrococcus* sp). All the test organisms were cultured in Nutrient broth and the 24hrs old cultures were used for the experiments.

Nathan's Agar well diffusion (NAWD) technique was followed for antibacterial susceptibility test [16]. Partial purification of the extracts was carried out [29]. After initial screening, the extracts showing activity obtained with butanol was fractionated using normal phase silica gel (200-400 mesh, LOBA CHEMIE, Mumbai, India) column chromatography employing a step gradient solvent system with increasing polarity.The fractions thus obtained were once again evaporated and concentrated. 5 mg of each of the dried fractions was dissolved in 70 µL Dimethyl sulfoxide (DMSO) and was again tested for antibacterial activity. After 24hrs of incubation at room temperature, the susceptibility of the test organisms was determined by measuring the radius of the zone of inhibition around each well. The Minimal inhibitory concentration (MIC) was determined for individual pathogenic strains that were found to be highly susceptible.

Results and Discussion

The ethyl acetate crude extract exhibited the highest activity against *Staphylococcus aureus* (4.5 mm), followed by *Bacillus cereus* and *Enterobacter aerogenes,* with inhibitory zones of 3.5 mm each. The extracts obtained with Acetone were found to inhibit *Vibrio cholerae* with a zone of inhibition of 3.5 mm followed by *Enterobacter aerogenes* with an inhibition zone of 2 mm. Ethyl acetate extracts were able to inhibit *Vibrio cholerae* with a zone of inhibition of 3 mm and the diethyl ether extracts against *Bacillus cereus* (2 mm), *Enterobacter aerogenes*, *Klebsiella pnemoniae* (Table. 1).

Table 1. Antibacterial activity of the crude extracts of *Cerithidea cingulata* against human pathogens and fish pathogens.

Solvents used	H	EA	AN	CH	A	DCM	M	DEE	T
Pathogens	Zone of Inhibition (mm)								
Bacillus cereus	1.5	3.5	2	1	-	1	T	2	--
Satphylococcus aureus	T	4.5	2	-	T	1.5	-	2.5	--
Vibrio cholerae	1.5	3	2.5	T	3.5	1	-	1.5	--
Enterobacter aurogenes	2	3.5	1.5	1	2	-	-	1	--
Klebsiella pneumoniae	1.5	2.5	1	T	-	-	-	1	T
Proteus mirabilis	T	2	1.5	1	1.5	-	-	1.5	-
Micrococcus sp	1	0.5	T	T	-	T	-	-	-
A. formicans	2.5	3	2	T	T	T	-	2.5	-
Aeromonas hydrophila	-	2.5	-	-	-	-	-	T	-
Serratia marcescens	-	3.5	2	-	2.5	T	-	1	-

H-Hexane, EA-Ethyl acetate, AN-Aceto nitrile, CH-Chloroform, A-Acetone, DCM- Dichloromethane, M-Methanol, DEE-Diethylether, T-Toluene

"--" – Nil; T- Trace

However, the extracts of *Pteria chinensis* obtained with acetone and chloroform exhibited higher activity and that obtained with toluene and butanol showed mild activities [3]. No inhibition zone was reported for the whole body extracts of *Cerithidea cingulata* [21]. However, one of the earlier works indicate that the hypobranchial glands of *Chicoreus virgineus* and egg capsules of *Rapana rapiformis* extracted with polar solvents like ethanol and methanol also have been reported to show wide spectral antibacterial activities [20].

Maximum zone of inhibition was exhibited against *Serratia marcescens* (3.5 mm) by the ethyl acetate extract followed by *Aeromonas formicans* (3 mm) and moderate zones of inhibition (2.5 mm) was shown against *A. formicans* by the extracts of hexane and against *A. hydrophila* by the extracts of ethyl acetate. *Proteus mirabilis* was moderately sensitive to extracts obtained with different solvents with a maximum zone of inhibition of 2 mm for the ethyl acetate extract. The crude extracts obtained with Acetone, Diethyl ether, Dichloromethane and Methanol showed inhibition against fish pathogens, *Serratia marcescens* and *Proteus mirabilis* (Table. 1). Interest in the chemistry of ophistobranch mollusks has been based on the finding that most of the molluskan secondary metabolites are derived from their diets [4]. They concentrate the metabolites from their highly specialized diets and incorporate into their own defensive strategies [8] which might be true for *Cerithidea cingulata* also, which mainly feeds on algae. Many biological studies of the natural products from these mollusks have therefore concentrated on potential ecological roles of the compounds. The present observation is further substantiated by the herbivorous mollusk, *Dolabella auricularia* which concentrates and stores selected algal metabolites [25]. Marine mollusks might provide potential for isolating compounds with specific activity against certain organisms or cell types [22].

The extracts obtained by column chromatography exhibited the highest activity against human pathogens when eluted with 100% ethyl acetate, which contradicts an earlier work stating that *Cypraea errones* has antibacterial activity at the non-polar end of the step gradient by the column-purified fractions [19]. The ethyl acetate fractions conferred inhibition against *Staphylococcus aureus* with zone of 5 mm, followed by *Bacillus cereus* (4 mm), *Vibrio cholerae* and *Enterobacter aerogenes* with zones of 3.5 mm each. (Table 2).

Table 2. Antibacterial activity of the Column purified extracts of *Cerithidea cingulata* against human pathogens and fish pathogens

Solvents used	H	80:20	60:40	40:60	20:80	EA	80:20	60:40	40:60	20:80	M
Pathogens	Zone of Inhibition (mm)										
Bacillus cereus	--	--	2	2.5	3.5	4.0	3	--	--	--	--
Staphylococcus aureus	--	--	3.5	4	4	5	4	--	--	--	--
Vibrio cholerae	--	--	2	2	2.5	3.5	2	--	--	--	--
Enterobacter aurogenes	--	--	--	2.5	3	3.5	3	--	--	--	--
Klebsiella pneumoniae	--	--	--	2.5	3	3.5	3	--	--	--	--
Proteus mirabilis	--	--	--	--	1	2	1.5	--	--	--	--
Micrococcus sp	--	--	--	--	0.5	1.5	1	--	--	--	--
A. formicans	--	--	--	1.5	2	3	2.5	--	--	--	--
Aeromonas hydrophila	--	--	--	2.5	3	3.5	3	1.5	--	--	--
Serratia marcescens	--	--	--	3	3	5	4	3.5	2	--	--

H-Hexane, EA-Ethyl acetate, M-Methanol.
"--" – Nil

The column purified ethyl acetate fractions were found to have pronounced antibacterial activities against both human and fish pathogens in comparison with the crude

extracts obtained with the respective solvents and a lesser degree of inhibition by the column-fractionated extracts. In comparison to the crude extracts it could be opined that the active compound may have degraded or modified during the fractionation process. The column purified extracts were able to cause a higher degree of inhibition in the case of human pathogens (Table 2). It is clear that out of the different fractions tested, the 100% ethyl acetate fractions were found to have a lower MIC, followed by the fractions with values of 1mg for all the pathogens (Table 3).

Table 3. Minimal Inhibitory Concentration of the column purified extracts of *Cerithidea cingulata* against human and fish pathogens

Solvents used	H	80:20	60:40	40:60	20:80	EA	80:20	60:40	40:60	20:80	M
Pathogens	Minimal Inhibitory Concentration (MIC) in mg										
Satphylococcus aureus	--	--	--	3	2	1	2	3	--	--	--
Vibrio cholerae	--	--	--	3	2	1	2	3	--	--	--
Aeromonas formicans	--	--	--	3	2	1	2	3	--	--	--
Serratia marcescens	--	--	--	3	2	1	2	3	--	--	--

mg- The minimal amount of the extract needed to inhibit a pathogen

It is clear that out of the different fractions tested, the 100% ethyl acetate fractions were found to have a lower Minimal Inhibitory Concentration (MIC) in inhibiting both the human and the fish pathogens, which is however higher than that of the 100% acetone fraction of *Pteria chinensis* for the pathogens *Serratia marcescens* (100 µg) and *Proteus mirabilis* (150 µg). A higher degree of inhibition was confined to ethyl acetate phases of *Cerithidea cingulata,* indicating that the substance involved in producing the antibacterial effect could be of medium polar type. The fractions of *Pteria chinensis* conferring antibacterial effect could be a medium-polar compound [3].

The wide spectrum antibacterial activity exhibited by the whole body extracts of *Cerithidea cingulata* indicates that it may possess biologically active metabolites. So, further fractionation and purification would reveal the nature of the compound for proper use as antibacterial agents. So there is an urgent need for the discovery of new and novel antimicrobial drugs to effectively combat not only the drug resistance but also the new disease producers. Therefore, the search for active drugs from alternative sources, including marine environment, obviously becomes imperative. New drug classes with novel mechanisms of action will create effective therapy at least for a period of time.

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