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Antimicrobial Activity of Sulfated Mucopolysaccharides [Heparin and Heparin-Like Glycosaminoglycans (GAGs)] from Cuttlefish *Euprymna berryi* Sasaki, 1929

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Abstract: The isolated heparin and heparin-like Glycosaminoglycans (GAGs) from the cuttlefish *Euprymna berryi* was studied for the antimicrobial activity. The bacterial strains such as (*Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escheriachia coli*, *Staphylococcus aureus*, *Shigella flexineri*) and fungal strains such as *Aspergillus fumigatus Fusarium* sp., *Cryptococcus neoformans*, *Microsporium* sp. and *Candida albicans* present study. various concentration (25, 50, 75 and 100%) used in this study. The heparin and heparin-like (GAGs) crude and purified sample showed activity against all the pathogenic bacterial strains. Whereas the antifungal activity in crude and purified sample was no activity against *Microsporium* sp. *Fusarium* sp. (crude) and *Microsporium* sp. (purified) respectively in all the concentration. The other all the fungal strains having activity in all the concentration. The activity of heparin and heparin-like GAGs extract was found to be higher in 100% concentration than the other concentration. In general the increasing concentration showed increasing activity of the extract. The heparin and heparin-like extract showing good antimicrobial activity are under going further analysis to identify the active constituents.

Key words: Heparin, glycosaminoglycans, antimicrobial activity

INTRODUCTION

Marine organisms are a rich source of structurally novel and biologically active metabolites. So far chemically unique compounds of marine origin with different biological activity have been isolated and a number of them are under investigation and/or are being developed as new pharmaceuticals (Da Rocha *et al*., 2001; Faulkner, 2000a, b; Schwartsmann *et al*., 2001).

Antibiotics are one of our most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction. However, over the past few decades these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not only because many of them produce toxic reactions but also due to emergence of drug resistant bacteria. It is essential to investigate newer drugs with lesser resistance. Systematic studies among various pharmacological compounds have revealed that any drug may have the possibility of possessing diverse functions and thus may have useful activity in completely different spheres of medicine (Sarkar *et al*., 2003). Therefore development of new technologies in search of novel bioactive compounds from marine sources will bring unique challenges and opportunities from seafood industry (Kim and Mendis, 2006). In the present an attempt has been made to study the antimicrobial activity of heparin and heparin-like GAGs of crude and purified samples isolated from the cuttlefish *E. berryi* was studied.

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MATERIALS AND METHODS

Antibacterial and Antifungal Activity

The animals were collected from the Mudasalodai landing centre, east coast of India, Tamil Nadu. The study was conducted in our laboratory situated at CAS in Marine biology, parangipettai. The crude and purified heparin and heparin-like GAGs was extracted from E. berryi using the method of (Holick *et al*., 1985) and were used to study their antibacterial and antifungal activities.

Preparation of Stock Solution

One milligram of each crude and purified heparin and heparin-like GAGs was dissolved in 2 mL of distilled water. From this 0.25, 0.50, 0.75 and 1.0 mL was taken and made up to 1.0 mL by adding distilled water to prepare various concentrations containing 125, 250, 375 and 500 µg of crude and purified sample corresponding to 25, 50, 75 and 100%, respectively.

Antibacterial Activity

Antibacterial activity was determined against *Bacillus subtilis*, *Escheriachia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Shigella flexineri* (obtained from Rajah Muthaiah Medical College, Annamalai University, Annamalai Nagar) using the paper disc assay method (El Masry *et al*., 2000). Whatman No. 1 filter paper disc of 6 mm diameter was sterilized by autoclaving for 15 min at 15 lbs (121°C).

Nutrient broth was prepared and sterilized in an autoclave at 15 lbs for 15 min. All the five bacterial strains were inoculated into the nutrient broth and incubated at 28±2°C for 24 h.

Nutrient agar medium was also prepared and transferred aseptically into sterile petridishes. The solidified nutrient agar medium in petridishes was incubated with the bacterial cells of 24 h old in the nutrient broth using the sterile cotton swab. The sterile discs were saturated with different concentrations of crude and purified samples (100, 75, 50 and 25%). The control with respective solvent (distilled water) was also prepared. The saturated disc was placed on the medium suitably spaced apart and the plates were incubated at 37°C for 24 h. The zone of growth inhibition, if any, was measured after the incubation period. Each extract was tested thrice for confirming the effect.

Antifungal Activity

Antifungal activity was tested against *Aspergillus fumigatus*, *Fusarium* sp., *Cryptococcus neofromans*, *Microsporium* sp. and *Candida albicans* (obtained from Rajah Muthaiah Medical College, Annamalai University, Annamalai Nagar) using the paper disc assay method as previously described in the antibacterial assay using the same stock solution for the four different concentrations.

The potato-dextrose broth was prepared and sterilized in an autoclave at 15 lbs. All the five fungal strains were inoculated into the potato-dextrose broth and incubated at room temperature for three days.

Potato-dextrose agar medium was also prepared and transferred aseptically into sterile petridishes. The solidified medium in the petriplates was incubated with the fungal cells of three days old in the potato-dextrose broth using sterile cotton swab.

The saturated discs (in different concentrations of crude and purified samples) were placed on the medium suitably spaced apart and the plates were incubated at 37°C for 24 h. The control with respective solvent (Distilled water) was also prepared. The zone of growth inhibition, if any, was measured after the incubation period. Each extract was tested thrice for confirming the effect.

RESULTS

Antibacterial Activity

The crude and purified sample of *E. berryi* showed activity against all pathogenic bacterial strains. The activity was higher in 100% concentration and lower in 25% concentration but activity was absent in control (Table 1, 2).

In 100% concentration, the highest inhibition zone was observed against *Shigella* sp. (5 mm) in crude sample; whereas in purified sample higher inhibition zone was observed against *Bacillus* sp. and *Staphylococcus aureus* (5 mm). The lowest activity, in terms of inhibition zone, was observed against *S. aureus* in crude sample (4 mm). But at the same time, 4.5 mm inhibition zone was recorded against *P. aeruginosa, E. coli* and *Shigella* sp. in purified sample and *B. subtilis* and *E. coli* in crude sample.

In 75% concentration, heparin and heparin-like GAGs showed maximum activity (4.5 mm inhibition zone) against *B. subtilis* and *S. aureus* in purified sample but in crude sample 4 mm inhibition zone was recorded against *B. subtilis*, *P. aeruginosa*, *E. coli* and *Shigella* sp. The lowest activity with 3.5 mm inhibition zone and moderate activity with 4 mm inhibition zone were observed against *E. coli* and *P. aeruginosa* respectively in the purified sample.

In 50% concentration, the maximum activity (4 mm inhibition zone) was recorded against *P. aeruginosa* in crude sample of heparin and heparin-like GAGs, but in purified, only 3.5 mm inhibition zone was observed against *P. aeruginosa* and *Shigella* sp. Minimum activity (3 mm inhibition zone) was showed against *B. subtilis*, *E. coli* and *S. aureus* in purified sample and 3.5 mm against *B. subtilis*, *E. coli* and *Shigella* sp. in crude sample.

At 25% concentration, the crude and purified samples showed more or less similar activity in all strains with the inhibition zone of 1.5 to 2.5 mm. The maximum of 2.5 mm inhibition zone was seen against *S. aureus* and *Shigella* sp. in crude sample and *B. subtilis* in purified sample. The moderate activity with 2 mm inhibition zone was recorded against *B. subtilis* and *P. aeruginosa* in crude sample and *P. aeruginosa*, *E. coli*, *S. aureus* and *Shigella* sp. in purified sample. The minimum activity (1.5 mm inhibition zone) was showed against *E. coli* in crude sample.

Table 1: Antibacterial activity of various concentrations of the crude heparin and heparin-like glycosaminoglycans from the whole body tissue of *E. berryi*

	Zone diameter (mm) crude			
	Concentration (%)			
Species name	25	50	75	100
Bacillus subtilis	2.0	3.5	4.0	4.5
Escheriachia coli	1.5	3.5	4.0	4.5
Pseudomonas aeruginosa	2.0	4.0	4.0	5.0
Staphylococcus aureus	2.5	3.0	3.5	4.0
Shigella flexineri	2.5	3.5	4.0	5.5

Table 2: Antibacterial activity of various concentrations of the purified heparin and heparin-like glycosaminoglycans from the whole body tissue of *E. berryi*

	Zone diameter (mm) purified			
	Concentration (%)			
Species name	25	50	75	100
Bacillus subtilis	2.5	3.0	4.5	5.0
Escheriachia coli	2.0	3.0	3.5	4.5
Pseudomonas aeruginosa	2.0	3.5	4.0	4.5
Staphylococcus aureus	2.0	3.0	4.5	5.0
Shigella flexineri	2.0	3.5	4.0	4.5

Antifungal Activity

The activity against the crude and purified heparin and heparin-like GAG sample was showed only by three and four fungal strains, respectively. In crude and purified samples there was no activity against *Microsporium* sp. And *Fusarium* sp. and *Microsporium* sp., respectively in all the concentrations tested (100, 75, 50 and 25%) (Table 3, 4).

At 100% concentration, the maximum inhibition zone of 5.5 mm was observed against *C. albicans* and *A. fumigatus* in crude and purified sample, respectively. The minimum of 3 mm inhibition zone was observed against *C. albicans* in purified sample. In purified sample 5 and 3.5 mm inhibition zone was observed against *C. neofromans* and *Fusarium* sp., respectively. At the same time 3.5 mm inhibition zone was observed against *A. fumigatus* and *C. neofromans* in crude sample.

In 75% concentration, highest activity (with 4.5 mm inhibition zone) was noted against *C. albicans* and *A. fumigatus* in crude and purified sample, respectively. Whereas 4 and 3 mm against *C. neofromans* and *Fusarium* sp., respectively in purified sample and 2.5 mm against *A. fumigatus* and *C. albicans* in crude and purified sample respectively was noticed. The lowest activity with only 2 mm inhibition zone was observed against *C. neofromans* in crude sample.

In 50% concentration, 3.5 mm inhibition zone was observed against *A. fumigatus* in purified sample, 3 mm against *C. albicans* in crude sample. In purified sample 2.5 and 2.0 mm inhibition zone was seen against *C. neofromans* and *C. albicans* and *Fusarium* sp. but in crude sample the lowest activity with 1.5 and 1 mm of inhibition zone was noted against *A. fumifatus* and *C. neofromans*, respectively.

At 25% concentration, the activity (in terms of inhibition zone) was observed between 0.5 and 1.5 mm. 1.5 mm inhibition zone was recorded against *A. fumigatus* in purified sample, 1 mm inhibition zone was noted against *C. albicans* and *C. neofromans*, *Microsporium* sp. and *Fusarium* sp. in crude and purified sample, respectively. The lowest activity with only 0.5 mm inhibition zone was recorded against *A. fumigatus* and *C. neofromans* in crude sample.

Table 3: Antifungal activity of various concentrations of the crude heparin and heparin-like glycosaminoglycans from the whole body tissue of *E. berryi*

	Zone diameter (mm) crude			
	Concentration (%)			
Species name	25	50	75	100
Aspergillus fumigatus	0.5	1.5	2.5	3.5
Fusarium sp.	-	-	-	-
Cryptococcus neofromans	1.5	2.5	4.0	4.0
Microsporium sp.	-	-	-	-
Candida albicans	1.0	3.0	4.5	5.5

Herpirn like glycosaminoglycans form the whole body tissue of *E. berry*

Table 4: Antifungal activity of various concentrations of the purified heparin and heparin-like glycosaminoglycans from the whole body tissue of *E. berryi*

	Zone diameter (mm) purified			
	Concentration (%)			
Species name	25	50	75	100
Aspergillus fumigatus	1.5	3.5	4.5	5.5
Fusarium sp.	1.0	2.0	3.0	3.5
Cryptococcus neofromans	1.0	2.5	4.0	5.0
Microsporium sp.	-	-	-	-
Candida albicans	1.0	2.0	2.5	3.0

DISCUSSION

Antimicrobial Activity of Heparin and Heparin-Like GAGs

Multicellular organisms express a blend of antimicrobial peptides. The various pathogenic bacteria are responsible for release of glycosaminoglycans from epithelia and connective tissues (Andersson *et al*., 2004). Heparin inhibits the growth of microorganisms gram-positive organisms are relatively susceptible and to this effect, gram-negative organisms are relatively resistant (Rosett and Hodges, 1980). The EDTA extract (polysaccharide) of *D. sibogae* gladius recorded 10 mm inhibition zone against *E. coli* and *K. pneumoniae*, 9 mm inhibition zone against *S. aureus* and 7 mm against *S. typhii*. Whereas the EDTA extract of *L. duvauceli* extract showed only low activity i.e., 5 mm against *P. aeroginosa*, 4 mm against *S. typhii* and *E. coli*. At the same time, the gladius extract of both the species showed no activity against *V. cholerae*. The polysaccharide extract from the gladius of *D. sibogae* recorded potent antibacterial activity against all the bacterial strain mentioned above and at the same time the polysaccharides of the *L. duvauceli* gladius extract recorded only low activity. The polysaccharide extract from the gladius of *L. duvauceli* showed antifungal activity against *A. fumigatus*, *A. flavus* and *Rhizopus* sp; whereas gladius extract of *D. sibogae* recorded the antifungal activity against *A. fumigatus* and *Rhizopus* sp. only. Whereas both the species showed no activity at all against *Candida* sp. (Barwin Vino, 2003). The antibacterial activity was predominant among cuttlebone extracts (using EDTA) of the cuttlefishes such as *S. aculeata* and *S. brevimana* against almost all the 9 pathogenic bacterial strains tested viz., *B. subtilis*, *E. coli*, *K. pneumoniae*, *S. aureus*, *V. parahaemolyticus*, *V. cholerae*, *S. typhii*, *P. aeroginosa* and *Shigella* sp. The activity was recorded in almost all the concentrations except in control. The antifungal activity of cuttlebone extract of *S. aculeata* and *S. brevimana* against four fungal strains such as *A. fumigatus*, *A. flavus*, *Candida* sp. and *Rhizopus* sp. showed the maximum activity of 100% and activity was found to be in an increasing order from the lower to higher concentration. On comparison the activity was higher in the cuttlebone extract of *S. aculeate* than *S. brevimana* (Mahalakshmi, 2003). In the present study, antibacterial activity of crude and purified heparin and heparin-like GAGs of *E. berryi* was studied in terms of the inhibitory zone produced by various bacterial and fungal strains and the diameter of inhibitory zone reported by the microorganisms was as follows: *E. coli* -4.5 mm (100% crude and purified), *P. aeruginosa* -5 mm (100% crude) and 4.5 mm (100% purified), *S. aureus* -4 and 5 mm (100% crude and purified) and *B. substilis* -4.5 mm (100%) and 5 mm (100% crude and purified) and antifungal activity in *C. albicans* -5.5 and 3 mm (100% crude and purified), respectively in which inhibition zone is higher than the above studies.

Likewise in the study of Sarkar (2003), on the antimicrobial activity of eight species of microorganisms, it was reported that the gram-positive Cocci (*S. aureus*, *S. epidermidis* and *C. albicans*) were more susceptible to heparin than were the gram-negative rods (*Citrobacter* sp., *K. pneumoniae*, *E. aerogenes* and *Enterobacter cloacae*) and an intermediate susceptibility of heparin was showed by the gram-negative rods *E. coli* and *P. aeroginosa*.

The activity of heparin and heparin-like GAGs extracts was found to be high in 100% concentration than the other concentrations. In general the increasing concentration showed increasing activity of the extracts. Therefore, it could be concluded that the antibacterial activity depends on the concentration. In the present investigation, the extract (both crude and purified) of heparin and heparin-like GAGs from *E. berryi* showed the antibacterial activity against all the five human pathogenic bacterial strains (*B. substilis*, *E. coli*, *P. aeruginosa*, *S. aureus* and *S. flexineri*) in increasing order in all the four concentrations (25, 50, 75 and 100%) tested. Likewise the antifungal activity was also studied in four different concentrations (25, 50, 75 and 100%) of the extracted heparin and heparin-like GAGs against five strains of fungi such as *A. fumigatus*, *Fusarium* sp., *C. neofromans*, *Microsporium* sp. and *C. albicans*. Though all the above fungi reported varying activities by showing difference in the

inhibition zone, the crude sample showed no activity in *Fusarium* sp. and *Microsporium* sp. and also the purified sample, against *Microsporium* sp. The result of the present provides larger information to the researchers involved in the filed of pharmacology and the scientists looking for useful drugs or drugs basic principles for the antibiotics and antifungal drugs against the human pathogens.

The uniqueness of the present study is that use of heparin and heparin-like extract obtained from cuttlefish, *E. berryi* is an efficient and cheap source of marine organism with remarkable antimicrobial activity. Detailed analysis at molecular level should be conducted to identify the active constituents that are responsible for this activity.

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