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RESEARCH ARTICLE

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COMPARATIVE STUDY ON THE ANTIMICROBIAL ACTIVITY, ANTI-OXIDANT, ANTI-DIABETIC AND ANTI-INFLAMMATORY PROPERTY OF FRESH WATER MOLLUSCS*(BELLAMYA BENGALENSIS*) AND MARINE MOLLUSCS (*SACCOSTREA CUCULLATA.)*

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Abstract

Freshwater and saltwater molluscs are potential uses to mankind. So, our present project work is "comparative study on the antimicrobial activity, anti-oxidant, anti-diabetic and anti-inflammatory property of fresh water mollusks *(Bellamya bengalensis*) and marine molluscs (*saccostrea cucullata)"***.** Antimicrobial activity of *Saccostrea cucullata* and *Bellamya bengalensis* was investigated against Gram positive bacteria including *Staphylococcus aureus* and *Bacillus subtilis* and Gram-negative bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*and *Proteus vulgaris.* Yeast including *Candida albicans* and fungi *Aspergillus flavus* were also used to test their antifungal activities. The body fluid of oyster showed highest antimicrobial activity against *B. subtilis* (20mm) and *C. albicans (17mm)* but *Bellamya sp*. shows highest antimicrobial activity against *S. aureus* and *C. albicans* 12mm and 12.6mm respectively with the MIC value as 20 µg/ml and 30mg/ml respectively. The MIC value of crude body fluid of oyster against *B. subtilis* and *C. albicans* were 5µg/ml and 4mg/ml respectively. Result also shows that the MIC value of both molluscs has lower than standard tested antibiotic (Ciprofloxacin). The present study indicated that body fluid of *S. cucullata* is a potential source of antifungal and antibacterial agents used as ethno-medicinal purpose. Therefore, body fluid of oyster and *Bellamya* sp. is alternative natural bio-product to cure some fungal (*C. albicans)* and bacterial (*B. subtilis, S. aureus)* disease rather than antibiotics. The body fluid of *S. cucullata* shows the higher anti-inflammatory, anti-diabetic and anti-oxidant property than *B. bengalensis*.

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Introduction:-

Bio-resources have been defined as living entities, which include genetic resources, organisms, and populations of living resources, with actual or potential uses to mankind. Specifically, Coastal ecosystem supports wild bioresources such as Estuaries and lagoons, Pelagic and Benthic ecosystems and marine fisheries. Aquatic bio-resources comprises of aquaculture, seaweeds, mangroves, corals. Aquaculture, also known as aqua farming, is the farming of aquatic animal and plants. Aquaculture comprises the cultivation of both fresh and saltwater populations under controlled conditions, and can be contrasted with commercial fishing, which is the harvesting of wild fish.

Saline water mollusks:-

Ocean is known as large biodiversity of fauna and flora (Kamboj, 1999). Therefore, the marine environment is an exceptional store house of different shellfish oranisms such as oyster, prawn, scallops, squids and octopus (Chandran, 2009). So, there is good opportunity for the discovery of new bioactive substances from marine and estuarine ecosystems.

Oysters in the oceans are a common sight and are virtually untapped resource of novel compounds. Oysters are bivalve soft bodied molluscs an shellfish under class Bivalve, mostly marine or estuarine in habit. As a sea food, oysters have been introduced and established in food permanently in at least 24 countries (Jannifer, 2005). In India, the common oyster species are *Saccostrea cucullata*, *Crassostrea madrasensis*, *Crassostrea gryphoides*, *Crassostrea rivularis* and *Crassostrea discoidea*. Out of these five dominant species, *S. cucullata* is the most abundant bivalve in the Hooghly estuary and normally grow on the hard substratum such as trunks of mangroves, concretes, embankments and piles and even on light house bases of Indian Sundarbans ecosystem (Mitra et al., 1993). Many studies reveled that molluscs has antitumor, anti-leukemic an anti-viral activities (Anand et al., 2002). This rich diversity to marine organisms assumes a greater opportunity for the discovery of new bioactive compound.

On the other hand, antibiotic resistance of different types of microorganism is the upcoming big problem for the treatment of human disease (Alanis et al., 2005). The increasing tendency of microbial infections, rapid immergence of drug resistant to antibiotics and quick evolution through mutation, poses an adverse effect on animal and required to develop new class of antibiotics. This has led to search of more antimicrobial substances from other natural sources including the aquatic environment (Kemper et al., 2008). There are about 7500 species of bivalves are identified (Gosling et al., 2003) which contain antibody-like materials that serve as defense from disease- causing organisms (Phillips et al., 1960). The molecular biological approach has proven more powerful than earlier protein/peptide based technique for the detection of novel conotoxins (Gosling et al., 2003). The screening of marine organisms, especially marine bivalves for therapeutic drugs are of greater interest now-a-days. Therefore, new natural compounds are required with less side effect and toxicological risks and no resistance developed by the pathogens (Chellaram, 2004).

Economic importance of oyster:-

- Oysters are nutritionally complete and are often referred to as the "milk of the ocean". Oysters contain protein, lipids, carbohydrates, different minerals (calcium, iron, copper etc.) and vitamins. However, it is a low-calorie food, at about 10 kcal per oyster.
- Oysters are low in cholesterol. They actually control the level of blood cholesterol i.e. the value of LDL and HDL. Oysters work to decrease your body's ability to absorb cholesterol from other foods. The percentage of fat in oyster flesh is very low (less than 2%) and contains almost no saturated fat making them a healthy heart.
- Oyster can supplies more than 100 percent of our daily zinc requirement, and many times more than the red meet.
- Oysters help boost of mental energy and maintain the activity of brain. Oyster protein contains tyrosine, which your brain converts into mentally energizing chemicals.
- Six oysters used as a supplement of iron and protein for daily diet. So, doctors recommended the oyster as a diet for patients with anemia.

These edible oysters are very popular as raw and processed food in South Indian States, Goa and in Europe, USA etc. Therefore, the aim of the present study was to evaluate the antimicrobial activity of the body fluid of *Saccostrea cucullata* against different pathogenic bacterial and fungal strains and also study the anti-oxidant, anti-diabetics and anti-inflammatory property of oyster body fluid.

Fresh water mollusks:-

As the global/country, population continues to grow, demand and production of food, especially aqua food from aquaculture will continue to be essential element in the future of our food security. The edible mollusks and mussel of the fresh water are widely distributed in different ponds and lakes. In the present study, several species of mollusks belonging to several families were collected and identified edible mollusc is considered parallel to the weed fishes here *Bellamya*. Sustainable utilization of molluscs is also essential because mollusc serve as food for many waterfowl, inhabiting the lake area. Compared to the fish farming the aquaculture of those Gastropods are still in this infancy in our country. There are two constrains in the development of shellfish's resources in our country, one sociological & other things are unscientific exploitation. Watching on the good economic potentiality of fresh water molluscs group we have to aware to the people, because it has been seen that it has good market demand, from various point of view. This creates a good entrepreneurship development, because it is a fact that spending less money, less time, lace place, less labor, & better to say very less annoying, a handsome output can be obtained from this sector. Basically molluscs are that type of animal whose all parts have several Industrial effects. So, why not will we show our interest in this under developed sector, particularly from the Industrial point of view because it is a sector where poorest poor people can be involved especially schedule tribes for their self development. In West Bengal many people mainly in the districts of West Medinipur, East Medinipur, Bankura, Howrah etc has taken as a profession of selling of Mollusc flesh or whole (Molluscs with shell), in the preparing of lime the role of shells of Molluscs have occupy a large space. In fresh water pearl production (derived mainly from *Lamellidens marginalis*) West Bengal creates a revolutionary effect among jewelry market. Many countries has developed good infrastructure & management on this sectors specially in value added edible product and its export, compared to them India is so lagging behind from them. Shell fishes are economically important animal biodiversity interacting intimately with local people of the village area. The diversity of species belonging to *Paratelphusa, Macrobrachium, Bellamya, Pila*, *Achatina*, *Lamellidens*, and *Novaculina* are used as folk medicines for the cure of a number of ailments such as rheumatism, cardiac diseases, controlling blood cholesterol and weakness of body (Mahata et al., 2002). The increasing tendency of microbial infections, antibiotic resistance property is greatest threats to microbial infection control that has generated the urgency to generate new antimicrobial compound. Peptides are the effective antibiotics to the combat increasing emergence of drug resistant bacteria. AMPs have broad-spectrum activity against a wide range of microorganisms including viruses, Gram-positive and Gram-negative bacteria, protozoa, yeasts and fungi. But no mass extract of fresh water molluscs (*Bellamya bengalensis*) have been reported as antibacterial activity against Gram positive bacteria including *Staphylococcus aureus* and *Bacillus subtilis* and Gram-negative bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Proteus vulgaris.* Yeast including *Candida albicans* and fungi *Aspergillus flavus* as it is more enteric pathogen of human and cattle. So, the locally available freshwater molluscs were chosen for present study with an objective to explore the antibacterial potentiality of its whole extract. Now in the present project work is "comparative study on the antimicrobial activity, anti-oxidant, anti-diabetic and anti-inflammatory property of fresh water molluscs *(Bellamya bengalensis*) and marine molluscs (*saccostrea cucullata)*.

Materials and Methods:-

Preparation of media, and antibiotics:-

Selective agar media (MHA) and Potato-dextrose agar (PDA) medium were purchased from HiMedia Laboratories Pvt. Ltd., India. Antibiotic and antifungal drugs were purchased from Cipla pharmaceuticals and Sun phrma, India.

Collection and Extraction of sample:-

The bivalves *(Saccostrea cucullata*)(4-6 cm) were collected from Frezargaunge river at Bakkhali part of Indian Sundarbans. The sample were kept in ice sterile bucket and taken to the laboratory for analysis within 6 hours of procurement. First of all, the oysters were rinsed thoroughly by sterilized distilled water then hard shells were punctured laterally and the body fluid was sucked by sterile syringe. The crude body fluid of *S. cucullata* was centrifuged at 15,000 rpm for 30 min at 4^0C, the supernatant was collected and stored at 20^0C.

Fresh water molluscs collection and extraction of sample:-

Fresh water molluscs*Bellamya bengalensis* was collected from a pond, near railway crossing, Panskura, Purba Medinipur. Collected samples were immediately stored into sterilizedice box and brought to the laboratory for determination of antibacterial activity of whole body extract.

Bacterial culture:-
The gram-positive bacteria such as *Staphylococcus sp., Bacillus subtilis* and gram negative *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and two fungus *Candida albicans* and *Aspergillus flavus* of pure culture were collected from collage laboratory. All the cultures were maintained and sub cultured on nutrient broth medium.

Determination of antimicrobial activity of body fluid of molluscs against selected pathogens:-
In vitro antibacterial activities of all aqueous body fluid extracts of both fresh water and saline water molluscs was determined by standard agar well diffusion assay (Nostro, 2000). Petri dishes (100 mm) containing 25 ml of Mueller–Hinton Agar and potato-Dextrose agar seeded with 100 µl inoculum of bacterial and fungal strain (Inoculums size was adjusted so as to deliver a final inoculums of approximately 10^6 CFU/ml for bacteria and $2x10^5$ for fungal spore). Media was solidified and respective plate was labeled for the spreading of fungi and bacteria. Wells were cut into solidified agar media with the help of sterilized cup-borer. 100 µl of each body fluid extract was poured in the respective wells and the plates were incubated at 37°C for bacteria overnight (Bhawna et al., 2010) and 30ºC for fungi for 96 hours (Ojha , 2013). Sterilized distilled water was used as negative control. The sensitivity was recorded by measuring the clear zone of growth inhibition on agar surface around the discs. Similarly, the sensitivity of antibiotics against tested pathogen was determined.

Determination of Minimum inhibitory concentration (MIC) value of lyophilized Body fluid:-
Minimum inhibitory concentration (MIC) was determined by agar and broth dilution methods (Chattopadhyay, 1998). A twofold serial dilution (0.5- 10 µg/ml) for bacteria and0.5-30 mg/ml for fungi) of the oyster body fluid was prepared in Mueller–Hinton Agar (bacteria) and potato-Dextrose agar (fungi). Similarly, for *Bellamya* sp. (5- 30 µg/ml) for bacteria and10-50 mg/ml for fungi was prepared in Mueller–Hinton Agar (bacteria) or potato-Dextrose agar (fungi). For agar dilution assay, previously prepared sensitivity plates, using serial two-fold dilutions of the body fluid, were spot-inoculated (2×10^6 CFU per/ml for bacteria and $2x10^5$ spores/ml per for fungi). The inoculated plates were then incubated at 37°C for 24 h (bacteria) and 30°C for 96 h (fungi). The lowest concentration of the plate or tube which did not show any visible growth after macroscopic evaluation was considered as the MIC (Chattopadhyay, 2001). Similarly, the MIC value of different antibiotics was determined.

Determination of anti-oxidant Property of Sample:-
The percentage of antioxidant activity (AA %) of each body fluid was assessed by DPPH free radical assay. The measurement of 2,2-diphenyl picryl hydrazyl (DPPH) radical traping was performed by Mensor et al., (2001). Molluscs lyophilized body fluid was reacted with stable DPPH radical in ethanol solution. The reaction mixture consisted of adding 0.5ml of lyophilized body fluid solution, 3ml of absolute ethanol and 0.3ml of DPPH radical solution 0.5ml in ethanol. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The color was changes from deep violet to light yellow and the absorbency was measured at 517nm after 100min of reaction using a UV-VIS spectrophotometer (DU 800; Beckman coulter, Fullerton, CA, USA). The mixture of ethanol (3.3ml) and Sample (0.5ml) was serving as blank. The control was prepared by mixing ethanol (3.5ml) and DPPH radical solution (0.3 ml).

Determination of anti - diabetic Property of Sample:-
Anti diabetic property of Sample was studied by Dhriti et al., (2014). 0.5 ml of standard starch was taken into different test tube and 0.5 ml of sample extracts was added into test tube and incubated at 37^0c for 3 min. After incubation, 1 ml of enzyme added into the mixture and incubated at room temperature for 15 min. One ml of DNS reagent was added to each test tube and then placed all the tubes in boiling water bath for 15 minutes. Then the absorbency was measured at 570 nm against blank.

Determination of anti-inflammatory property of Sample:-
The assay was done by following methods (Suganya et al., 2014) at 660 nm. The reaction mixture consists of lyophilized body fluid solution at concentration 1000 µg /ml and 1% aqueous solution bovine albumin fraction. The pH of the reaction mixture was adjusted to 6.5 using 1 (N) HCl and incubated at 37^oc for 20 minutes then heated at 57^oc for 30 minutes. The denaturation process is stopped by cooling the sample and finally turbidity was measured using colorimeter. The standard substance used as aspiri and controle was measured without lyophilized body fluid solution.

Statistical analysis:-
All the data was analyzed statistically using SPSS-10.0. Each sample was analyzed and data were represented as mean ±SD.

Results:-

Table 1:- Antimicrobial activity of body fluid (100µl) of *S.cucullata* and *B. bengalensis* against eight selected pathogens

Sample	Bacteria						Fungi	
	E.coli (mm) mean±SD	*K.pneumonia* (mm) mean±SD	*S.aureus* (mm) mean±SD	*B.subtilis* (mm) mean±SD	*P. vulgaris* mean±SD	*P.aurogenosa* (mm) mean±SD	*A.flavus* (mm) mean±SD	*C.albicans* (mm) mean±SD
S. cucullata	18±0.6	11±0.6	15±0.9	20±1.4	10±0.3	13±1.0	11±1.0	17±1.2
B. bengalensis	9.0±0.7	9±0.5	12±0.5	11±0.5	9.1±0.7	9.5±0.9	11±0.7	12.6±0.8
Sterile water (Control)	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0

Table 2 :- MIC value of lyophilized aqueous body fluid of *S. cucullata* showed against *B. subtilis* (µg/ml) and *C.*albicans (mg/ml)

Organism	Lyophilized body fluid	Zone of inhibition (mm) mean±SD
Bacillus subtilis	10	20±0.9
	5	15±0.5
	4	0.0
	2	0.0
	1	0.0
	0.5	0.0
C. albicans	30	17±1.1
	20	15±1.2
	10	14±1.1
	5	12.5±1.0
	4	12.1±1.0
	2	0.0
	1	0.0
	0.5	0.0

Table 3:- MIC value of lyophilized aqueous body fluid of *Bellamiya bengalensis* against *S. aureus* (µg/ml) and *C.albicans* (mg/ml)

Organism	Lyophilized body fluid	Zone of inhibition (mm) mean±SD
S. aureus	30	20±0.9
	25	15±0.5
	20	11±0.6
	15	0.0
	10	0.0
	5	0.0
C.albicans	50	17±1.1
	40	15±1.2
	30	12±1.1
	25	0.0
	20	0.0

	15	0.0
	10	0.0
	5	0.0

Table 4:- The sensitivity of some pathogenic bacteria through MAR (multiple antibiotics resistance) test (100µg/ml)

Antibiotics	Zone of inhibition (cm) against Bacteria					
	E .coli Mean±SD	***B. subtilis*** Mean±SD	***K. pneumonia*** Mean±SD	***P. vulgaris*** Mean±SD	***S. aureus*** Mean±SD	***P. aeruginosa*** Mean ±SD
Azithromycin	-	3.2±0.15	3.3±0.057	2.5±0.11	2.2±0.057	2.9±0.15
Amoxicillin	2.3±0	1.4±0	1.8±0.15	2.6±0.11	1.8±0.1	2.06±0.11
Ampicillin	2.5±0.057	-	1.6±0.17	2.01±0.057	1.6±0.057	1.9±0.1
Chloramphenicol	-	2.4±0.057	2.5 ±0	1.2±0.1	2.7±0.057	-
Ciprofloxacin	2.6±0.057	3.6±0.057	3±0.057	1.9±0.35	3.3±0.11	2.1±0.3
Cefuroxime	-	-	1.4±0	-	1.6±0.057	1.5±0.05
Doxycyclin	-	2.4±0.057	2.6±0.057	-	3.5±0.25	2.9±0.057
Ofloxacin	1.9±0.2	2.4±0.057	2.9±0.057	-	2.8±0	1.2±0.057

Table 5:- MIC value of different antibiotics against bacteria

Antibiotics	MIC value (µg/ml) of antibiotics against Bacteria					
	E.coli	***S. aureus***	***B.subtillis***	***K.pneumonia***	***P.aurogenosa***	***P. vulgaris***
Azithromycin	-	25	1	0.25	1	0.5
Amoxicillin	2	25	50	10	2	10
Ampicillin	20	30	40	10	8	5
Chloramphenicol	-	25	5	10	5	-
Ciprofloxacin	8	25	5	1	0.1	0.5
Cefuroxime	-	25	1.5	-	75	3

Table 6:- Sensitivity of Antibiotics against Fungi (250 mg/ ml)

Name Of Antibiotics	Zone of inhibition (cm) of antibiotics against Fungi	
	Aspergillus flavus	Candida albicans
Flucanazole	1.9 ±0.17	1.1 ± 0.057
Griseofulvin	2.3 ± 0.2	2.1 ±0.057

Table 7:- MIC values of antibiotics (Fluconazole & Griseofulvin) against Fungi

Name of fungi	Concentration	Antimicrobial zone(cm)	
		Griseofulvin	Flucanazole
Candida albicans	250 mg/ml	2.1	1.1
	150 mg/ml	1.5	-
	100mg/ml	1.3	-
	50mg/ml	-	-
Aspergillus flavus	250mg/ml	2.3	1.9
	150 mg/ml	1.9	1.2
	100mg/ml	-	-
	50 mg/ml	-	-

Table 8:- Determination of anti-inflammatory activity of body fluid by inhibition albumin denaturation method

OD value	Sample name	
	Saccostrea cucullata	*Bellamya* bengalensis
Blank	0.00	0.00
Test	0.11	0.19
Control	0.06	0.33

Table 9:- Determination of anti-diabetic activity of body fluid by alpha-amylase method

OD value	Sample name	
	Saccostrea cucullata	*Bellamya* bengalensis
Blank	0.00	0.00
Test	0.02	0.12
Control	0.10	0.19

Table 10:- Determination of anti-oxidant activity of body fluid by DPPH method

OD value	Sample name	
	Saccostrea cucullata	*Bellamya* bengalensis
Blank	0.00	0.00
Test	0.09	0.90
Control	0.12	0.19
(AA)%	55	NA

The Scavenging activity percentage (AA %) $=100-\frac{[(Abs_{sample}- Abs_{blank}) \times 100]}{Abs_{control}}$

Discussion:-

Since antimicrobial resistance is a global public - animal health concern, there is a growing interest in marine ecosystem to find new antimicrobial agents which will be essential drugs for human and animal health and welfare. In the present study, a pronounced antimicrobial activity of the body fluid of *S. cucullata* has been observed against some bacterial and fungal strains. Maximum antimicrobial activity was observed against *B. subtilis* and *C. albicans* compare to other microorganism. The MIC value of lyophilized body fluid of *S. cucullata* exhibit inhibitory activity mostly against bacterial strain *B. subtilis* and fungal strain *C. albicans* but the value is lower (4.0mg/ml) than standard antibiotics (Table- 2&4) . The research shows that the medicinal value of the gastropod *S.cucullata* body fluid and muscle may be due to high quality of antimicrobial compounds. The flesh of oyster was used widely in India and China as a fisher folk medicine to treat several liver diseases like jaundice, hepatitis-A and B (Wang et al., 2006). Fresh water snail (*Bellamya bengalensis*) consumption is a source of low cost protein in under developed countries like India, Bangladesh, Vietnam, etc. In many countries, it is consumed in raw form. Present study reveal that fresh water molluscs also have anti bacterial and anti fungal activity but comparatively lower than studied oyster as *Saccostrea cucullata.* Therefore, body fluid of oyster and *Bellamya* sp. is alternative natural bio-product to cure some fungal (*C. albicans)* and bacterial (*B. subtilis, S. aureus)* rather than antibiotics. The body fluid of *S. cucullata* shows the higher anti-inflammatory, anti-diabetic and anti-oxidant property than *B. bengalensis.*

Conclusion:-

Body fluid of oyster (*Saccostrea cucullata*) and *Bellamya* sp. is alternative natural bio-product to cure some fungal (*C. albicans)* and bacterial (*B. subtilis, S. aureus)* rather than antibiotics.

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