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Antimicrobial activity of *Prosopis stephanin* and *Lantana camara* on isolated *Vibrio* from aquaculture in Delvar region, Bushehr, Iran

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ABSTRACT: Vibriosis is one of the most prevalent bacterial fish diseases worldwide. On the other hand, use of antimicrobial agents in aquacultures may be able to induce antibiotic resistance. In the present study totally 87 water, sediment and shrimp samples were collected from two aquaculture in Delvar in Bushehr province during May-August 2014. The samples were cultivated on TCBS agar medium and incubated at 370C for 24 hrs. Then the pure isolates were identified using API Kit 20E. Furthermore, effect of methanol, acetone and aqueous extract of *Lantana camara* and *Prosopis stephanin* leaves were evaluated on the isolates using well diffusion agar technique and MIC of the herbal plant for each organism were calculated. Finally the isolates were identified using molecular technique. The results indicated that totally three V*ibrio* species were detected which is confirmed using biochemical and molecular technique and they were belong to *V. coralliilyticus* strain ATCC BAA-450 (96% Identities), *V. alginolyticus* strain l67 (96%Identities), *V. harveyi* (94% Identities). Aqueous extract of *Prosopis stephanin* had no effect on isolates. Methanol extract of *Prosopis stephanin* and Acetone extract of *Lantana camara* had inhibitory effect against all of the isolates but Acetone extract of *Prosopis stephanin* had maximum antimicrobial effect on *V. harveyi* with minimum inhibitory concentration 0.005 ± 0.001 gr/ml. It could be concluded that *Lantana camara* and *Prosopis stephanin* with antimicrobial effect could be use for remedy of vibriosis in shrimp aquacultures.

Keywords: Shrimp aquaculture, Vibriosis, *Lantana camara*, *Prosopis stephanin*

INTRODUCTION

Shrimp culture industry is a fast producer food product in the world wild (Gopal *et al.,* 2005). Development of shrimp culture industry has been accompanied with development of diseases such as Vibriosis. Vibriosis has been an important cause of production loss due to bacterial disease in shrimp farms in south Iran in recent years (Raissy *et al.,* 2011; Mahbob, Ahmed & Paul 2011). Treatment of shrimp diseases with antibiotics has led to the emergence of antibiotic-resistant (Seong Wei, Musa & Wee 2010; Nadirah, Wee & Najiah 2013; Ramesh *et al.,* 2014). On the other hand, some of the medicinal plants contain compounds that can cure infectious diseases through antibacterial, antifungal and antiviral activities.

Several studies have investigated antimicrobial agents found in medicinal plants that grow in Asia. Akhila *et al.* (2013) reported that water, ethanol, methanol and ethyl acetate extracts of *Pajanelia longifolia* had antibacterial activity against *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Escherichia coli*, *Bacillus subtilis*, *Proteus mirabilis*, *Lactobacillus casei* and *L. fermentum*) Al-Judaibi *et al.,* 2014). Because of the importance pathogenic *Vibrio* species in aquaculture and development of antibiotic resistance in this species, Replacing one drug the nature of herbal medicines and antibacterial properties is particularly important in the prevention of resistant strains and risks of transfer resistance genes to consumers. In present study, we compare the antibacterial activity of some plants grown in Bushehr province. Specifically, we investigate extracts of *Prosopis stephanin* and *Lantana camara* with regard to activity against *Vibrio* spp. isolated from Delvar in Bushehr province.

METHODS AND MATERIALS

A. Sample collection

In total 87 water, sediment and shrimp samples were collected from aquatic shrimp cultures of three regions such as Delvar, in Bushehr, Iran during May to August in 2014 (Table 1).

B. Isolation and identification of Vibrio spp.

Water and sediment samples were analyzed directly and added into Alkaline pepton water (APW), Pretreatment of the shrimp samples was carried on by removing the microbiota from the surface of the bodies using alcohol (70%). Afterward, the samples have been taken from hepatopancras and added into the APW medium.

Table 1: Locations of sample collection, the results of the three is repeated.

Location	Shrimp	Sediment	Water	Total sample
Delvar14	5	5	5	15
Delvar18	4	5	5	14
Total	9	10	10	29

After 24 hours 0.1 ml of the APW suspensions was cultivated onto thiosulfate citrate bile sucrose agar medium and the plates incubated at 37°C for 24 hours (Melo *et al.,* 2011). The suspected green and yellow colonies growth on TCBS medium were phenotypic identified using Gram stain, catalase, oxidase, production of hemolysins and API 20E kits (Noriega-Orozco *et al.,* 2007).

C. Preparation of Plant Extracts

About 50 g of shade dried coarse powder of each selected plants were exhaustively extracted at room temperature for 48 hrs with 300ml of various solvents of increasing polarity: acetone, methanol and water. After filtration, the solvent was removed by evaporation using a rotary evaporator under reduced pressure at temperature below 55°C (Lilybeth, Olowa & Olga 2013).

D. Minimum Inhibitory Concentration (MIC) for Anti-Vibrio

The sensitivity of each leaves extract of the plant was determined using the agar well diffusion method. Bacterial cultures in tryptic soy broth (TSB) (Merck, Germany) was adjusted to 0.5 McFarland and incubated for 18 h at 30°C (Irobi, Young & Anderson 1996). The extract was diluted into different concentrations viz. 1, 0.5, 0.25, 0.63, 0.031, 0.016, 0.0078 and 0.0039 gr/ml. A sterile 6 mm diameter cork borer was used to bore wells into the Mueller- Hinton agar medium. The wells were then filled up with approximately 100µl of the extract solution and incubated at 37ºC for 24 hours. The test was continued out in triplicates. At the end of the incubation period, inhibition zone formed on the agar were measured in mm using a transparent ruler (Nadirah *et al.,* 2013).

E. Molecular identification of isolated bacteria

Identification of isolated bacteria was verified by Gene sequencing of 16SrRNA. To perform the experience DNA was extracted from the isolated strains using High Pure Template DNA polymerase chain reaction (PCR) kit according to the supplier's instructions (Sinagen company). The purity of the extracted DNA was assessed based on absorbance of the extracted DNA at 260 and 280 nm wavelengths, then the purity was calculated based on absorbance ratio of 260/280nm. The extracted DNA with ratio (260/280nm) 1.9≤ corresponding to 121µg DNA ml^{-1} was used for amplification of 16S rRNA by PCR. Amplification of 16S rRNA was performed using universal Forward and Reverse 5'-3' 27F (GAGTTTGATCCTGGCTCAG) and 5'-3' 1392R (ACGGGCGGTGTGTRC) (Mirbakhsh *et al.,* 2014).

PCR amplification conditions on an Eppendrof thermocycler were as follows: 95°C for 4 min, followed by 35 cycles of 95°C for 40 s, 56°C for 30 s, and 72°C for 40 s, with a final extension at 72°C for 5 min and stored at 4°C. All PCR products obtained were run on a 1.5% (w/v) agarose gel with a 1kb DNA ladder. PCR products were electrophoresed at 95V for 20 min; DNA was visualized using ethidium bromide and photographed. After visualization of pure DNA bands, the PCR products have been sent to Macrogen Company for DNA sequencing. The 16S rRNA sequenced data for bioinformatic applications were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) in order to identify each respective 16S rRNA gene amplicon.

F. Statistical analysis

Statistical analysis of data collected was carried out using the Analysis of Nonparametric tests (Kruskal wallis) at the 0.05 level of significance with Statistical Analysis System program.

RESULTS AND DISCUSSION

A. properties of sample location

Physical and chemical properties of sample collection locations were shown in Table 2. As mentioned in this table physical and chemical characters of these regions are approximately were differed in the months of June, July and August.

Table 2: Physico- chemical properties of sample location.

Location	Oxygen Dissolved (mg/L)	Temperature (°C)	NaCl concentration(mg/L)	pH
Delvar 14	6-7	29-32.5	52.3	8.3
Delvar 18	4-6.5	31-33	43.7	8.5

B. Phenotypic identification of isolated strains

The results obtained from phenotypic identification of the isolates using biochemical tests (Table 3) and API 20E kit (Table 4) indicated that the isolates were *Vibrio cholera*, *Vibrio alginolyticus* and *Vibrio vulnificus.*

Table 3: Biochemical characteristics of Vibrio spp. Isolates, 1: M2MT, 2: 13DT, 3: 19DM.

Number of colony	Shape	TSI	Catalase	Oxidase	production of Hemolysin	Gram stain	Color of colony in TCBS
1	Rod bacilli	A/A	+	+	β	-	yellow
2	Rod bacilli	A/A	+	+	α	-	yellow
3	Rod bacilli	A/A	+	+	α	-	yellow

Table 4: Biochemical characters of *Vibrio* spp. isolates based on API 20E kit, 1: M2MT, 2: 13DT, 3: 19DM, O-nitrophenyl-b-D-galactopyranoside, Arginine dihydrolase, Lysine decarboxylase, Ornithine decarboxylase, Citrate, Production of hydrogen sulfide, Urease, Tryptophan deaminase, Indole, Voges-Proskauer, Gelatinase, Fermentation of glucose, Fermentation of mannose, Fermentation of inositol, Fermentation of sorbitol, Fermentation of rhamnose, Fermentation of sucrose, Fermentation of melibiose, Fermentation of amygdalin, Fermentation of arabinose.

No	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TAD	IND	VP	GEL	GIU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
1	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	+	-	-	-
2	-	-	+	-	+	-	-	-	+	-	-	+	+	-	-	-	+	-	+	-
3	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	+	-	+	-

C. Minimum Inhibitory Concentration (MIC)

It was found that the leaves aqueous extract of of *Prosopis stephanin* had no effect on isolates. Acetone extract of *Prosopis stephanin* had maximum antimicrobial effect on isolates number three with minimum inhibitory concentration 0.0052 ± 0.0013 gr/ml.

Table 5 show the means diameter of antimicrobial activity extracts. The minimum inhibitory concentrations (MIC) for the aqueous, methanolic and acetonic extract of *Prosopis stephanin* and *Lantana camara* are shown in Table 6. Fig. 1 show compare the minimum inhibitory concentration of plant extracts on isolates.

Table 5: The means diameter of antimicrobial activity of aqueous, methanolic and acetonic leaves extract by well diffusion methods (mm), Mean±SD. The results of the three is repeated, 1: M2MT, 2: 13DT, 3: 19DM.

Isolate	Zone of Inhibition (mm) ± SD					
	Extract					
	Aqueous		Methanol		Acetone	
	Prosopis stephanin	*Lantana camara*	*Prosopis stephanin*	*Lantana camara*	*Prosopis stephanin*	*Lantana camara*
1	00.00	00.00	20.33 ± 0.57	10.50 ± 0.50	00.00	17.30 ± 0.30
2	00.00	12.93 ±0.11	32.83 ± 0.28	00.00	17.93 ± 0.11	12.93 ± 0.11
3	00.00	00.00	14.10 ± 0.10	00.00	19.16 ± 0.28	13.20 ± 0.34

Table 6: Minimum Inhibitory Concentration (MIC) of *Prosopis stephanin* and *Lantana camara* leaves extracts against isolates (mg/ml) by well diffusion methods, Mean±SD, The results of the three is repeated, 1: M2MT, 2: 13DT, 3: 19DM.

Isolate	MIC (mg/ml) ± SD					
	Extract					
	Aqueous		Methanol		Acetone	
	Prosopis stephanin	*Lantana camara*	*Prosopis stephanin*	*Lantana camara*	*Prosopis stephanin*	*Lantana camara*
1	000.000	000.000	0.062 ± 0.000	1.000 ± 0.000	000.000	0.166 ± 0.041
2	000.000	0.041±0.010	0.166± 0.041	000.000	0.166 ± 0.041	0.166 ± 0.041
3	000.000	000.000	1.000 ± 0.000	000.000	0.005 ± 0.001	0.666± 0.166

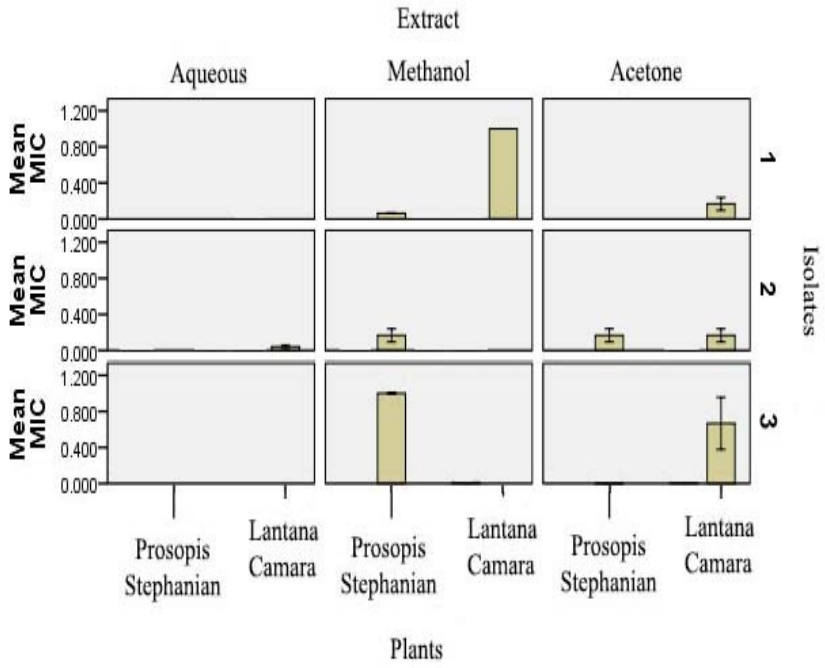

Fig. 1. Compare the minimum inhibitory concentration of plant extracts on isolates,1: M2MT, 2: 13DT, 3: 19DM.

D. Molecular Identification of antibiotic resistant Vibrio spp. Isolates

The isolated bacteria were 96, 96 and 94% identical with *Vibrio alginolyticus* strain L67, *Vibrio coralliilyticus* strain ATCC BAA-450 and *Vibrio harveyi* (Fig. 2).

In this study, the results obtained from phenotypic and molecular identification were differ, but both methods were concured to each other concerning to isolates of *Vibrio* genus. *Vibrio coralliilyticus* Species is a new type toward previous studies in shrimp aquaculture in bushehr province.

Statistical analysis of the data in the present study was done based on the effect of plant extract (MIC) against each isolate using non- parametric the Kruskal-Wallis test. The statistic test illustrated the significant results with p value <0.05 for each extract on each isolate. On the over hand, compared plants extract effect on isolates. In this study Screening assay of the ethanolic, methanolic and aqueous extracts of *Prosopis stephanin* and *Lantana camara* leaves showed promising antibacterial activities against all *Vibrio* strains tested. Aceton extract of *Prosopis stephanin* had maximum antimicrobial effect on *V. harveyi* with minimum inhibitory concentration 0.005 ± 0.001 gr/ml.

The largest inhibition zone was observed from methanol extracts against *V. coralliilyticus* (32.83 mm). Aqueous extracts of both plants showed less anti-vibrio activity compared to the extracts obtained from other solvents.

Fig. 2. Gel electrophoresis of 16srRNA gene of Vibrio spp. Isolates, M: Marker 1kb, Line 1: *Vibrio alginolyticus* strain L67, Lin2: *Vibrio coralliilyticus* strain ATCC BAA-450, Line 3: *Vibrio harveyi*.

CONCLUSIONS

It could be concluded that *Prosopis stephanin* and *Lantana camara* have potentially good antimicrobial efficacy against of microbes examined. In this study, we obtained results of the antimicrobial activity of the two native species from bushehr province, contributing to increasing the knowledge of the plant used to in traditional medicine and could be the basis for further studies to isolate the active compounds of the studied plants and evaluate their effectiveness against other microorganisms.

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