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Original Article

Antibacterial potential of selective aqueous plant extracts against *Vibrio parahaemolyticus*

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ABSTRACT

To evaluate the anti-bacterial activity of the selected plants, *Lawsonia inermis* (Lythraceae), *Phyllathus niruri* (Phyllanthaceae) and *Eucalyptus kitsoniana (*Myrtaceae) against *V.parahaemolyticus*. Which is a marine bacterium, responsible for disease outbreak affecting commercial production of shrimp species especially in *Penaeus monodon*. In this study, twenty seven colonies of *V. parahaemolyticus*, were isolated by cultivating in TCBS agar. The colonies were analyzed by bio chemical assay and PCR method confirming that it was *V. parahaemolyticus*. The aqueous plant extracts were prepared from the selected plant samples and evaluated for their antibacterial activity against *V. parahaemolyticus*. The antibacterial activity was tested by using the disk diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods.

Keywords: PCR, MIC, MBC, *Vibrio parahaemolyticus*, *Penaeus monodon*

INTRODUCTION

Aquaculture is the fastest growing industry. To meet the demand for sea food and aqua products throughout the world, traditional aqua farming gave way to intensive aquaculture. Physiological stress and suppression of immune system which leads to disease out breaks in aquaculture is often attributed to intensive aquaculture. A serious economic loss of shrimp production was reported due to bacterial disease [1] [2]. Shrimp farmers and shrimp hatcheries have reported high mortality in their culture due to Vibrio species, especially *V. parahaemolyticus*. Several *Vibrio* spp. are present as natural biota of fish and shellfish [3] [4] [5] [6]. Some of the *Vibrio* species such as *V. harveyi* and *Vibrio parahaemolyticus* are the major infections causing bacteria in shrimp [2] [7] [8]. One of the major difficulties in biochemical identification of *V. parahaemolyticus*, because these bacterium has involving the sucrose fermentation [9] in addition PCR technique is the advanced method to identifying the *V. parahaemolyticus*.

This bacterium possesses a regulatory gene, toxR, which is present in all strains irrespective of their Kanagawa reactivity [10]. PCR based on toxR and on a chromosomal locus of unknown function reported to be specific for *V. parahaemolyticus* [11] has been found to be useful for confirmation of this species [9].

Antibiotics are used in large quantities, even when the presence of pathogens is not evident in the hatcheries. Antibiotics, which have been used in large quantities, are in many cases not only ineffective in culture tanks but also increase the virulence of pathogens. The use of heavy antibiotics in aquaculture may cause development of antibiotic resistance strains among pathogens infecting cultured animals. It has led to an increase in antibiotic resistance *vibrios*, and presumably other bacteria also having multiple antibiotic resistant and to an increase in more virulent pathogens. The expanding bacterial resistance to antibiotics has become a growing concern worldwide [12]. Hence, the use of non-chemotherapeutic methods for disease treatment in aquaculture are beneficial to the industry. Medicinal plants could be those alternatives because most of them are safe, cost less and affect a wide range of antibiotic resistant microorganisms [13] [14]. In fact medicinal plants have already been used as remedy for many infectious diseases [15] since ancient time. According to Abutbul *et al.*, 2005, plants having antibacterial properties are

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potentially beneficial to aquaculture. Recently, a number of studies have been carried out to determine the anti-bacterial properties of plants [17] [18]. A vast number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds [19]. Medicinal plant extracts offer considerable potential for the development of new agents effective against infections currently difficult to treat [20].

The present study was carried out to test the antibacterial efficacy of plant extracts of three plants: *Lawsonia inermis* (Lythraceae), *Phyllathus niruri* (Phyllanthaceae) and *Eucalyptus kitsoniana (*Myrtaceae) against a shrimp pathogen (*V. parahaemolyticus*).

MATERIALS AND METHODS

Collection of Post larvae: The samples were collected from 10 hatcheries around the coastal regions of Nellore (AP), Kakinada (AP) and Poducherry. From each hatchery around 500 numbers of post larvae were collected containing diseased post larvae rearing tanks. The samples were oxygenated and packed in polypropylene bags and then transported to the laboratory. Each batch was maintained in separate 20 litre culture tanks. The water maintained between temperature was 25 to30^{0}C, salinity 15 to 20ppt, pH 7.8 to 8.4 and dissolved oxygen at 3 to 5 ppm continuous aeration was provided.

Sample preparation and Isolation of *Vibrio* species: 10 to 15 post larvae from each tank were taken aseptically. The hepato-pancreas, carapace with gills and mid gut regions with pleopods of the post larvae was dissected out. The dissected parts were homogenized in 100µl of 9% saline. All the samples were diluted serially and 100 µl aliquots were spread on a plate containing Thiosulfate Citrate Bile Salt Sucrose agar (TCBS, SRL, Bombay, India), After 24 hours of incubation on TCBS agar, the number of colonies and morphology of colonies were recorded for all samples. Each green colony was sub cultured onto Tryptic Soy Agar (TSA, SRL, Bombay) with 2% sodium chloride and incubated at 30^{0}C.

Biochemical assay to identify the *V.Parahaemolyticus:* Green colonies from each sample were purified and sub cultured on TSA with 2% sodium chloride. The identification of *V.Parahaemolyticus* from the sub culture was carried at using biochemical reactions assay which included MR-VP reaction, growth in the absence of NaCl and ONPG [21].

MR-Voges-Proskauer (VP): Test assay was performed by using a culture grown in MR – VP medium and incubated at 37^{0}C for 48 hr [22].

Growth in the absence of NaCl: Cells grown in the absence of NaCl in 1% tryptone broth (Oxoid Ltd., Basingstoke, England) were used to determine the requirement for Na+ .

ONPG: For the ONPG (O-nitrophenyl-betaD-galactosidase) test, a 24 hr – old culture was inoculated into tubes containing saline solution. The ONPG disks were added and the tubes were incubated at 37^{0}C for 24 hr. Positive test was indicated by a development of yellow color.

Confirmation of *V.Parahaemolyticus* DNA extraction: Test culture was grown in 5.0 ml Muller Hinton broth at 37^{0}C overnight. The DNA isolated from the cell suspension was used as template. The sequence from toxR gene was used for detection of *Vibrio parahaemolyticus.* Specific primers (R: 5'- GTCTTCTGACGCAATCGTTG - 3' and F: 5' – ATACGAGTGGTTGCTGTCATG – 3') correlated to *Vibrio parahaemolyticus* toxR region with 50% GC contents were used [2,10]. The primer was obtained from Bio-serve India Pvt. Ltd. Hyderabad, India.

Polymerase Chain Reaction (PCR): PCR-Protocol was carried out using 0.5 ml PCR tubes. The total volume of reaction mixture was 22.0 µl which consisted of 15 µl sterile distilled water, 2.0 µl 10x PCR buffer with MgCl2, 2 µl 25mM deoxyribonucleotide phosphate, 1 µl of each primer, 0.5 µl of 0.5 units Taq DNA and 1.5 µl template DNA. The cycling conditions were as follows; pre denaturation at 94°C for 5 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min and final extension at 72°C for 7 min at the end of 25 cycles.

Electrophoresis: The PCR products were run on 1.6% agarose gel (SRL) prepared with 1x Tris-Borate- EDTA (TBE) and Ethidium bromide (0.5µg/ml) added before pouring to the gel plate. 15µl of end PCR products were loaded into sample wells in the TBE loaded gel tank. 100 volt power was supplied for 30 mins. Then the gel was visualized and photographed under UV transilluminator.

Sub culture: Identified colonies were sub cultured onto Tryptic Soy Agar (TSA, SRL, Bombay) with 2% sodium chloride and incubated at 30^{0}C for further use.

Plant collection: The three plants, *Lawsonia inermis, Phyllathus niruri* and *Eucalyptus kitsonia* were collected randomly in and around Vellore Dt.,

Tamil Nadu, India during the month of January, 2014. Fresh plant material was washed with distilled water and shade dried at room temperature. The plant material was authenticated and voucher specimens were kept at Dept., of Zoology, Thiruvalluvar University, Serkadu, VelloreDt, Tamilnadu, India. The shade dried plant materials were powdered by using electric blenders.

Preparation of plant extract: The herbal extracts were prepared at the rate of 1g/5ml of solvent in 250ml Erlenmeyer flasks with intermittent shaking. The flasks were closed with cotton plug aluminium foil with intermittent shaking for 48hours at room temperature filtered through Whatman filter paper No.1 and then concentrated by using a rotary evaporator at temperature levels between 40-50ºC. The extracts were preserved in airtight bottles and stored at 4 °C until further use.

Antibacterial Activity Study

Agar well diffusion assay: The selected plant extracts were used for studying their antibacterial activity. A loop of bacterial strain from the confirmed determined subculture was inoculated into 30 ml of nutrient broth in a conical flask and incubated for 24 hours to get pure culture. 100µl pure culture inoculums ($1x10^6$ cfu/ml) from nutrient broth were inoculated into sterile Muller Hinton agar plates. For agar well diffusion method, three wells were prepared in the plates with the help of a cork-borer (5 mm). 100mg, 50mg and 25mg of the crude plant extract and blank (aqueous) were introduced into the three wells separately. 15µg streptomycin added into the well served positive control. The plates were incubated overnight at 37°C. Antimicrobial activity was determined by measuring the diameter of zone of inhibition. All the samples were tested in triplicates and results were recorded.

Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC): *Lawsonia inermis, Phyllathus niruri* and *Eucalyptus kitsoniana* plant extracts showing considerably potent antibacterial activity. The plant extracts that were found to be effective, as antimicrobial agents, were later tested to determine the MIC and MBC values for the selected strain. MIC was determined using broth dilution method. The plant extracts were diluted with Muller Hinton broth to give the final concentrations of 100, 50, 25, 12.5, 6.2, 3.1mg/ml. 100 µl (10^6 CFU/ml). The pure *Vibrio parahaemolyticus* strains from nutrient broth were inoculated into 5ml of Muller Hinton broth with different concentration plant extract tubes. The tubes were incubated under aseptic condition at 37°C for 24 hrs. Streptomycin which was used as the positive control (15µg) was added into 5 ml of Muller Hinton broth. The minimum bactericidal concentration (MBC) was defined as the minimal concentration of the plant extract which completely inhibited the visible growth of the bacteria on solid media in the petriplates that were incubated at 37ºC for 24 hrs.

RESULTS AND DISCUSSION

Aquaculture is one of the fastest growing food production sectors in the world. Disease outbreak in culture ponds and pathogenic infections in hatcheries result in low production and high economic loss in aquaculture. Such infectious diseases can spread through food, water and other exogenous sources. Care is to be taken to produce high quality post-larvae, which are stocked in nurseries and grow out ponds for more sustainable production. Pathogenic bacteria affected diseased shrimp shows identical symptoms like reddishness and mass mortality. *V.parahaemolyticus* is a major pathogen causing severe survival loss especially in *Penaeus monodon* [23]. In the present study, *P.monodon* post larvae samples were collected from 10 affected hatcheries located around Kakinada (AP), Nellore (AP) and Puducherry. The selective media TCBS agar was used to detect the causative vibrio colonies (Table: 1). In the TCBS agar plates 51 green colonies (Table: 1) were identified. The green colonies indicate the presence of *V.parahaemoluticus*, *V.mimicus* and *V.damsella* species. Each green colony from the TCBS media were sub cultured into TSA agar media for biochemical assay and PCR tests for identification of species.

Fifty one subcultures were subjected to bio chemical tests which included MR-VP reaction, Growth in absence of NaCl and ONPG and PCR methods. Eleven colonies showed positive result by MR-VP test assay, which indicated that it was *V.damsella* species (Table: 2,3&4). About thirteen colonies showed positive result in growth in absence of NaCl and ONPG test, and hence were identified as *V.mimicus* species (Table: 2,3&4). Twenty seven colonies were identified as *V. parahaemolyticus* which was confirmed by PCR method (Table:2, 3 &4). The colonies were confirmed as *V.parahaemolyticus* by PCR method because the gene sequence had the toxR gene at 368 bp chromosomal locus which is specific for this species. Also stated that the PCR method targeted to the *tox*R gene can be used as a method for identification of *V. parahaemolyticus* at the species level [24] [10]. The *tox*R targeted gene PCR method is more sensitive in detecting *V. parahaemolyticus* than the method based on

isolation and biochemical identification [25]. The total number of colonies identified from the 10 samples was 213; among which 27 colonies were *V.parahaemolyticus* (12%) colonies.

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources [26]. India is a land of rich biodiversity. The total number of lower and higher plants in India is about 45,000 species [27]. Many plants have been sources of medicines since ancient times. Yet a scientific study of plants to determine their antimicrobial active compounds [28] is a comparatively new field. Numerous surveys on biological important medicinal plants had been made in United States and in many countries throughout the world. Such study had demonstrated the wide occurrence of active compounds in higher plants [28, 29]. In this connection this study was carried out the efficacy of antibacterial activity of the aqueous extract of *Lawsonia inermis, Phyllathus niruri* and *Eucalyptus kitsoniana* against the 27 identified colonies of *V.parahaemolyticus.* The antibacterial activity was assessed by the presence or absence of zone of inhibition, MIC and MBC methods. These plants showed significant antibacterial activity.

The aqueous leaf extract of *Lawsonia inermis*, showed maximum zone inhibition (13.5 mm at 100 mg concentration) against *V.parahaemolyticus* pathogen, 7.0mm at 50mg concentration and 3.0mm at 25 mg concentration on the agar well diffusion plates. The minimum inhibitory concentration of *Lawsonia inermis* was recorded between 6.3mg/mL and 12.5mg/mL. The Minimum bactericidal concentration (MBC) of the *Lawsonia inermis* recorded between 12.5mg/ml to 25mg/ml.
The plant extract of *Phyllanthus niruri* showed maximum zone inhibition against *V.parahaemolyticus* at 100mg concentraion and 50 mg concentration were 5.5mm and 2.5mm respectively. The minimum inhibitory concentration (MIC) of plant extract was recorded between 25mg/mL and 50mg/mL. The minimum bactericidal concentration (MBC) of the plant was recorded between 25mg/ml to 50mg/ml.

The leaf extract of *Eucalyptus kitsoniana* recorded maximal zone of inhibition at 24.5 mm at 100 mg concentration against *V.parahaemolyticus*, 14.0mm at 50mg concentration and 9.0 mm at 25 mg concentration on agar well diffusion plates. The minimum inhibitory concentration (MIC) of plant extract was as 6.25mg/ml and the MBC value was 6.25 between 12.5. This result showed that the *Eucalyptus kitsoniana* plant extract showed more antibacterial activity against *V.parahaemolyticus* when compared to other plant extracts.

The lowest concentration (highest dilution) of the extract that produced no visible growth (no turbidity) in the first 24 h when compared with the control tubes was considered as initial MIC. The dilutions that showed no turbidity were incubated further for 24 h at 37 °C. The lowest concentration that produced no visible turbidity after a total incubation period of 48 h was regarded as final MIC. MBC value was determined by sub culturing the test dilution [which showed no visible turbidity] into freshly prepared nutrient agar media. The plates were incubated further for 18-42 h at 37 °C. The highest dilution that yielded no bacterial colony on the nutrient agar plates was taken as MBC.

CONCLUSIONS

V. parahaemolyticus causes enormous economic loss in *P.monodon* aquaculture. This study concluded that *E.kitsoniana* and *L.inermis* aqueous extracts have a significant antimicrobial effect against bacterial shrimp pathogens especially *V.parahaemolyticus.* Moreover, these natural antimicrobial agents were more effective in controlling the disease causing bacterial strains and suppressing the development of resistant strains. These plant extracts can be used as a possible alternative source to synthetic antibiotics. However, further extensive research and development work should be undertaken on the solution of the active components of these plant extracts for their better economic and therapeutic utilization. In vivo clinical testing is also essential to confirm in vitro results. More research on the production antibiotics from medicinal plants can provide an effective solution for treatment of shrimp bacterial diseases, thus avoiding the use of synthetic antibiotics.

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Table 1: Results on TCBS Agar

Sample	K1	K2	K3	K4	N1	N2	N3	P1	P2	P3
No. of yellow colonies	20	22	24	20	18	19	17	28	30	25
No. of green colonies	5	5	6	6	4	5	4	4	6	6

Table 2: The biochemical and PCR test for green color colonies: Sample from Kakinada (AP)

Samples	MR-VP Test	Growth in 0% NaCl	ONPG Test	PCR	Vibrio spp.
K1 i	+	-	-	-	*V.damsela*
K1 ii	-	-	-	+	*V.parahaemolyticus*
K1 iii	-	-	-	+	*V.parahaemolyticus*
K1 iv	-	+	+	-	*V.mimicus*
K1 v	-	-	-	+	*V.parahaemolyticus*
K2 i	-	+	+	-	*V.mimicus*
K2 ii	-	-	-	+	*V.parahaemolyticus*
K2 iii	+	-	-	-	*V.damsela*
K2 iv	-	+	+	-	*V.mimicus*
K2 v	-	-	-	+	*V.parahaemolyticus*
K3 i	-	-	-	+	*V.parahaemolyticus*
K3 ii	-	-	-	+	*V.parahaemolyticus*
K3 iii	-	+	+	-	*V.mimicus*
K3 iv	+	-	-	-	*V.damsela*
K3 v	-	-	-	+	*V.parahaemolyticus*
K3 vi	-	-	-	+	*V.parahaemolyticus*
K4 i	-	+	+	-	*V.mimicus*
K4 ii	-	+	+	-	*V.mimicus*
K4 iii	-	-	-	+	*V.parahaemolyticus*
K4 iv	-	-	-	+	*V.parahaemolyticus*
K4 v	-	+	+	-	*V.mimicus*
K4 vi	-	-	-	+	*V.parahaemolyticus*

Table 3: The biochemical and PCR test for green color colonies: Sample from Nellore (AP)

Samples	MR-VP Test	Growth in 0% NaCl	ONPG Test	PCR	Vibrio spp.
N1 i	-	-	-	+	*V.parahaemolyticus*
N1 ii	+	-	-	-	*V.damsela*
N1 iii	+	-	-	-	*V.damsela*
N1 iv	-	-	-	+	*V.parahaemolyticus*
N2 i	-	+	+	-	*V.mimicus*
N2 ii	-	-	-	+	*V.parahaemolyticus*
N2 iii	-	-	-	+	*V.parahaemolyticus*
N2 iv	-	+	+	-	*V.mimicus*
N2 v	-	-	-	+	*V.parahaemolyticus*
N3 i	-	+	+	-	*V.mimicus*
N3 ii	-	-	-	+	*V.parahaemolyticus*
N3 iii	-	-	-	+	*V.parahaemolyticus*
N3 iv	+	-	-	-	*V.damsela*

Table 4: The biochemical and PCR test for green color colonies: Sample from Puducherry

Samples	MR-VP Test	Growth in 0% NaCl	ONPG Test	PCR	Vibrio spp.
P1 i	+	-	-	-	*V.damsela*
P1 ii	-	-	-	+	*V.parahaemolyticus*
P1 iii	-	-	-	+	*V.parahaemolyticus*
P1 iv	+	-	-	-	*V.damsela*
P2 i	+	-	-	-	*V.damsela*
P2 ii	-	-	-	+	*V.parahaemolyticus*
P2 iii	-	-	-	+	*V.parahaemolyticus*
P2 iv	+	-	-	-	*V.damsela*
P2 v	-	+	+	-	*V.mimicus*
P2 vi	-	-	-	+	*V.parahaemolyticus*
P3 i	-	+	+	-	*V.mimicus*
P3 ii	-	+	+	-	*V.mimicus*
P3 iii	+	-	-	-	*V.damsela*
P3 iv	-	-	-	+	*V.parahaemolyticus*
P3 v	-	-	-	+	*V.parahaemolyticus*
P3 vi	-	-	-	+	*V.parahaemolyticus*

Table 5: Antibacterial activity of *Lawsonia inermis* by zone of inhibition, MIC and MBC method

	Zone of inhibition			MIC	MBC
	100mg	50mg	25mg		
K1	12.0mm	6.5mm	2.0mm	6.25	12.5
K2	12.5mm	6.5mm	2.5mm	12.5	12.5
K3	12.5mm	7.0mm	2.0mm	12.5	12.5
K4	13.0mm	5.0mm	2.5mm	12.5	12.5
N1	12.0mm	5.5mm	3.5mm	12.5	25.0
N2	13.5mm	6.0mm	2.5mm	6.25	12.5
N3	12.5mm	6.5mm	3.5mm	12.5	25.0
N1	13.0mm	6.0mm	2.5mm	12.5	12.5
N2	12.5mm	6.0mm	3.5mm	12.5	12.5
N3	12.5mm	5.0mm	2.5mm	6.25	12.5

Table 6: Antibacterial activity of *Phyllathus niruri* by zone of inhibition, MIC and MBC method

	Zone of inhibition			MIC	MBC
	100mg	50mg	25mg		
K1	5.0mm	2.5mm	no zone	25	25
K2	4.5mm	2.5mm	no zone	25	25
K3	5.5mm	2.0mm	no zone	25	50
K4	5.0mm	2.0mm	no zone	25	25
N1	5.5mm	2.5mm	no zone	25	50
N2	4.5mm	2.0mm	no zone	50	50
N3	4.0mm	no zone	no zone	25	25
N1	5.0mm	3.0mm	no zone	25	50
N2	4.5mm	2.0mm	no zone	50	50
N3	5.5mm	2.0mm	no zone	25	25

Table 7: Antibacterial activity of *Eucalyptus kitsoniana* by zone of inhibition, MIC and MBC method

	Zone of inhibition			MIC	MBC
	100mg	50mg	25mg		
K1	22.0mm	10.5mm	6.0mm	6.2	6.2
K2	24.5mm	12.5mm	7.5mm	6.2	6.2
K3	20.5mm	10.0mm	6.0mm	12.5	12.5
K4	23.0mm	11.0mm	7.5mm	6.2	12.5
N1	21.0mm	11.5mm	6.5mm	6.2	6.2
N2	22.5mm	12.0mm	7.5mm	6.2	6.2
N3	22.5mm	13.5mm	8.5mm	6.2	6.2
N1	23.0mm	14.0mm	7.5mm	6.2	12.5
N2	24.5mm	14.0mm	9.5mm	6.2	6.2
N3	22.5mm	12.0mm	7.5mm	6.2	12.5

REFERENCES

1. Takahashi Y et al. Pathogenicity and characteristics of Vibrio sp. isolated from diseased postlarvae of kuruma shrimp, Penaeus japonicus Bate. Bull. Jpn. Soc. Sci. Fish. 1985; 51: 721-730.
2. Lightner DV. Diseases of cultured penaeid shrimps. In: Mc Vey, J.P. (Ed.), CRC Handbook of Mariculture, 2nd ed., CRC Press, Boca Raton, 1993; pp. 393 – 486.
3. Vanderzant C et al. Microbial flora of pond reared brown shrimp (Penaeus aztecus). Applied Microbiology. 1971; 21, 915 – 921.
4. Colwell RR. Vibrios in the Environment. Wiley, New York. Colwell, R.R., Spira, W.M.,. In: Barua, D., Grunough III, W.B. (Eds.), Cholera. Plenum Medical book Co., New York, 1992; pp. 107 – 127.
5. Ruangpan L, Kitao T. Vibrio bacteria isolated from black tiger shrimp, Penaeus monodon Fabricus. Journal of Fish Diseases. 1991; 14, 383 – 388.
6. Otta SK et al. Bacterial flora associated with shrimp culture ponds growing Penaeus mondon in India. Journal of Aquaculture in Tropics 1999; 14, 309 – 318.
7. Jiravanichpaisal P, Miyazaki T. Comparative histopathology of vibriosis in black tiger shrimp, Penaeus monodon. In: Shariff, M., Subasinghe, R.P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture II. Fish Health Section, Asian Fisheries Society, Manila, Philippines, 1995; pp. 123 – 130.
8. Lavilla-Pitogo C.R. Bacterial diseases of penaeid shrimps: an Asian view. In: Shariff, M., Arthur, J.R., Subasinghe, R.P. (Eds.), Diseases in Asian Aquaculture II. Fish Health Section, Asian Fisheries Society, Manila, 1995; pp. 107 – 121.
9. Karunasagar I et al. Rapid detection of Vibrio parahaemolyticus from fish by polymerase chain reaction (PCR). In: Flegel, T.W., MacRae, I.H. (Eds.), Diseases in Asian Aquaculture III. Fish Health Section, Asian Fisheries Society, Manila, 1997; pp. 119 – 122.
10. Kim Y.B et al. Identification of Vibrio parahaemolyticus strains at the species level by the PCR method targeted to the toxR gene. Journal of Clinical Microbiology 1999; 37, 1173 – 1177.
11. Lee C.Y et al. Sequence of a cloned pR72H fragments and its use for detection of Vibrio parahaemolyticus in shell fish with PCR. Applied and Environmental Microbiology. 1995; 61, 1311 – 1317.
12. Gardam M.A. Is methicillin-resistant *Staphylococcus aureus* an emerging community pathogen? A review of the literature. Can. J. Infect. Dis. 2000; 11:202-211
13. Nimri LF et al. Antibacterial Activity of Jordanian medicinal plants. *Pharm. Biol.* 1999; 37: 196-201.
14. Karaman S, Kocabas YZ. Traditional medicinal plants of K. Maras (Turkey). *The Sciences* 2001; 1:125-128.
15. Rios JL, Recio MC. Medicinal plants and antimicrobial activity. J. Ethnopharmacol. 2005; 100. 80 - 84.
16. Abutbul S et al. Screening of desert plants for use against bacterial pathogens in fish, Isr. J. Aquac. Bamidgeh. 2005; 57. 71-80.
17. Alzoreky NS, Nakahara K. Antibacterial activity of extracts from some edible plants commonly consumed in Asia,Int. J. Food Microbiol. 2003; 80.223-230.
18. Kim JS, Kim J. The inhibitory effect of natural bioactives on the growth of pathogenic bacteria, Nutr. Res.Pract. 2007; 1: 273-278.
19. Mahady G.B. Medicinal plants for the prevention and treatment of bacterial infections. Curr. Pharm. Des. 2005; 11:2405-2427
20. Iwu, MW et al. New antimicrobials of Plant Origin. *In*: J. Janick. Ed. *Perspectives on New Crops and New Uses.* ASHS Press, Alexandria, VA. 1999; pp. 457-462.
21. Farmer J.J et al. The genera Vibrio and Photobacterium. In: Balows, A., Truper, H.G., Schleifer, K.H. (Eds.), The Prokaryotes, vol. 2. Springer-Verlag, New York, 1992; pp. 2952 – 3011
22. Twedt RM et al. Vibrio. In Compendium Methods for the Microbiological Examination of Foods. 2nd Ed., (Marvin LS, Editor). Washington DC: APHA Inc 1984
23. Jayasree S. Identification of immune cells interacting with Vibrio spp. and its in vitro post-phagocytic killing mechanism of haemocytes in the penaeid shrimp, Penaeus indicus H. Milne Edwards. J Fish Dis.2009; 32(4):359–365.
24. Lin Z et al. Vibrio parahaemolyticus has a homolog of the Vibrio cholerae toxRS operon that mediates environmentally induced regulation of the thermostable direct hemolysin gene. Journal of Bacteriology. 1993; 175: 3844 – 3855
25. Dileep V et al. Application of polymerase chain reaction for detection of *Vibrio parahaemolyticus* associated with tropical seafoods and coastal environment. Letters in Applied Microbiology
26. Cragg GM, Newman DJ. Medicinals for the millennia. *Ann NY Acad Sci*.2001; 953:3-25.
27. Jain SK. Ethnobotany and research on medicinal plants in India. Ciba Found Symp. 1994; 185:153-164.
28. Madhumitha G, Saral AM. Antimicrobial activity of successive extracts of *Thevetia neriifolia. Asian Journal of chemistry*. 2009; 21: 2471-2472.
29. Vinothkumar P et al. Invitro Antibacterial Activities of *Picrorhiza kurroa* rhizome extract using Agar well diffusion method. *Int.J.Curret Pharmaceutical Sci*. 2010. 2(1):30-33.