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RESEARCH ARTICLE

Anti-microbial activity of spices black cardamom, mustard seed and liquorice against *V. cholera, V. parahaemolyticus* and *V. vulnificus*

■ **NAVEEN KUMAR SHUKLA AND UMA SHANKAR**

SUMMARY

There are several water-borne diseases which are highly pathogenic for humans. Among all, *Vibrio* genus plays a major role. *Vibrio cholerae*, a gram negative anaerobe, is one of the most ancient and major water borne pathogen which causes fatal disease, cholera. This study chiefly focuses on the natural or plant derived products which could be used against *Vibrio*. In this work, 10 pond water samples were collected and investigated for presence or absence of pathogenic *Vibrio* species. A rapid method, multiplex PCR,of identification has been applied for determining the species. Out of 10, 8 samples showed presence of *Vibrio* species in them when cultured on selective media. The identified isolates of *V. cholera, V. parahaemolyticus* and *V. vulnificus* were then tested against three common spices, black cardamom *(Amomum subulatum)*, mustard seed *(Brassica nigra)* and liquorice *(Glycyrrhiza glabra)* , extracts for their antibacterial property using agar well diffusion method. The antimicrobial assay result of spices showed a maximum activity by aqueous extract liquorice *(Glycyrrhiza glabra)* of 23 ± 0.52 (mm) against *V.vulnificus* followed by mustard seed *(Brassica nigra)* of 22.1 ± 0.60 (mm) against *V.vulnificus.*

Key Words : Black cardamom, Mustard seed, Liquorice, mPCR, TOX gene, *Vibrio* species, Antibacterial activity

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The increasing population of the coastal zone introduces a high percentage of untreated sewage into the sea, and this form of pollution is probably responsible for the greatest number of human morbidities and mortality worldwide (Roszak and Colwell, 1987). In particular, waterborne infections like typhoid fever, cholera, dysentery and traveller's diarrhea, caused by different types of bacterial pathogens pose a major public health hazard (Hunter, 1997), especially in developing countries. The spectrum of waterborne infections is also expanding, and many infectious diseases once believed to be conquered are on the rise (Marshall *et al.*, 1997). In order to protect public health, regular monitoring of waterborne pathogens is required. However, the

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deficiency of precise and cost-effective diagnostic methods is a major obstacle in the prevention and control of infections and outbreaks transmitted by waterborne pathogens.

Plants have been a valuable source of natural products for a long period of time to maintain human health, especially with more intensive studies in the last decade for natural therapies (Gislene *et al*., 2000). Spices have been used for not only flavor and aroma of the foods but also to provide antimicrobial properties (Nanasombat *et al*., 2002). Spices may contribute piquancy of foods and beverages (Perween and Nazia, 2006). In addition to these spices are some of the most commonly used natural antimicrobial agents in foods. Some of the natural compounds found in various spices possess antimicrobial (Indu *et al*., 2006). Over 50 per cent of all modern clinical drugs are of natural product origin whereas the natural products play an important role in drug development programs in the pharmaceutical industry (Baker *et al*., 1995). In recent years, focus on plant research has increased all over the world. Collected evidences showed immense potential of medicinal plants used in various traditional systems, for their biological activities and antioxidant principles (Farombi, 2003; Gilani *et al*., 2005 and Pan *et al*., 2004).

MATERIAL AND METHODS

Chemicals and media :

All the bacterial culture media (TCBS Agar and Tryptone Soya Agar) used were from HiMedia, India. The chemicals were from Merck. Primers and enzymes were purchased from Sigma-Aldrich, St. Louis, USA and used as recommended by the manufacturers.

Isolation of *Vibrio* species from pond water :

The water samples collected from ponds of five locations in Lucknow, India. The samples were collected in air tight, sterilized bottles and immediately brought to lab for isolation of microbes. *Vibrio* species were isolated on selective media- Thiosulfate-citrate-bile salts sucrose agar (TCBS agar) media and appearance of green and yellow colonies were observed. Based on their morphological appearance, cultures were further maintained on Tryptone Soya Agar slants after incubating at 37 °C for 24 h.

DNA extraction and quantification :

Tryptone soya broth was prepared and inoculated under aseptic condition with the isolated *vibrio* species. DNA was extracted from bacteria grown in the culture broth (TS broth), using phenol:chloroform, Tris HCl, Sodium dodecyl Sulphate, Tis-EDTA. The DNA was electrophoresed on 0.8 per cent agarose gel and its size was estimated using the DNA marker of integrated DNA technologies (IDT) 1 kb. The isolated DNA was quantified using UV spectrophotometer (Systronics-2205).

Amplification and identification of *Vibrio* species using multiplex PCR :

Identification of *Vibrio* species for each water sample was done using species specific primers for five different species of *Vibrio* (*V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, *V. mimicus and V. alginolyticus*) including both pathogenic and non-pathogenic *Vibrio* species. They are targeted at a species-specific tox gene region of the *Vibrio*. Table A lists the primers used for the amplification of these genes (Singh *et al*., 2015).

The PCR reactions were performed as instructed by manufacturer. A 20µl reaction mixture containing 10X Taq DNA polymerase buffer with 2mM $MgCl_2$, 2.5 mM dNTPs, 5µM primers (Sigma-Aldrich), Taq DNA polymerase (Sigma-Aldrich), template DNA and sterile distilled water was prepared and mixed gently. Temperature cycle of 35 cyclers was used for amplification of TOX gene with denaturation at 94ºC for 30 sec, annealing at 60ºC for 30 sec, extension at 72ºC for 1 min and final extension at 72ºC for 7 mins.

Table A : List of primers used for amplification and amplicon size in thermal cycler

Target species	Primer	Sequence(5'-3')	Amplicon size
Universal primer	Universal F.W primer	CAGGTTTGYTGCACGGCGAAGA	-
V. alginolyticus	Reverse primer	GATCGAAGTRCCRACACTMGGA	144
V. vulnificus	Reverse primer	GTACGAAATTCTGACCGATCAA	412
V. cholera	Reverse primer	AGCAGCTTATGACCAATACGCC	375
V. mimicus	Reverse primer	YCTTGAAGAAGCGGTTCGTGCA	177
V. parahaemolyticus	Reverse primer	TGCGAAGAAGGCTCATCAGAG	96

Preparation of spice extract :

Three spices namely black cardamom (*Amomum subulatum*), mustard seed (*Brassica nigra*) and liquorice (*Glycyrrhiza glabra*) were collected from vendors in Lucknow. Extract was prepared at 10 per cent (w/v) of spices and respective solvents (RANKEM Chemicals). Solvents used were acetone, methanol and distilled water extract preparation of spices.

Maintenance of bacterial culture :

The stock culture of the identified three *Vibrio* species (*V. cholera, V. parahaemolyticus* and *V. vulnificus*) was maintained at 4°C in Tryptone soya agar slants.

Antimicrobial assay of spices against *V. cholera, V. parahaemolyticus* and *V. vulnificus* :

The antimicrobial analysis method was performed using agar well diffusion method (Bauer *et al.*, 1966) to evaluate the antimicrobial properties of three spices: Black cardamom (*Amomum subulatum*), mustard seed (*Brassica nigra*) and liquorice (*Glycyrrhiza glabra*). Nutrient agar plates were prepared to evaluate the antimicrobial activity of spices extract, against selected *Vibrio* species. Bacterial overnight grown culture was uniformly spread on nutrient agar plates, after five minutes 6mm diameter well was bored in the plates. 20µl of spices extract, standard antibiotic solution (as positive control) and solvents (as negative control) was poured into the well. The plates were incubated at 37^0C for 24hr. and after incubation plates were observed for the zone of inhibition (mm).

RESULTS AND DISCUSSION

A total number of 10 water samples were collected from different regions of Uttar Pradesh. Out of 10 samples, result revealed that, 8 locations showed presence of *Vibrio* spp. with appearance of green and yellow colonies of varied morphology (Table 1).

The amplification products showed presence of *V. cholera, V. parahaemolyticus or V. vulnificus*. The samples which showed their presence were cultured and maintained for antibacterial assay of spices. Results of antibacterial activity showed by the extracts of spices are shown in Table 2.

The *Vibrio* infection in the farmed shrimps was reported during the post-monsoon season, when the water temperature was increasing. Elevation in water temperature and constant alkaline pH may help the spread of *Vibrio* in the farm setting. Numerous studies have already been done to identify many *Vibrio* species on the basis of biochemical tests, Biology GN techniques,

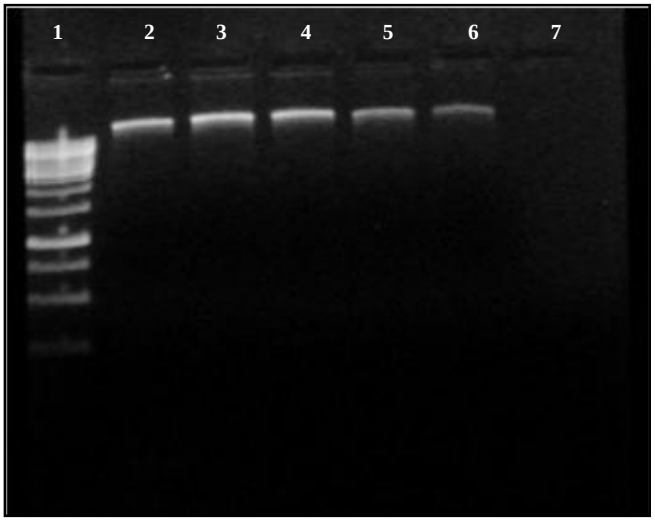

Fig. 1(a) : A 0.8 per cent agarose gel, showing isolated genomic DNA stained in ethidium bromide and visualized under UV light ; Lane 1: ladder; Lane 2-6: genomic DNA samples of bacteria

Table 1 : Presence of *Vibrio* spp. with appearance of green and yellow colonies of varied morphology on locations

Sr. No.	Location	Type	Region	G_C*	Y_C*	G_C and Y_C
1.	Indira Nagar	Pond water	Lucknow.	-	-	-
2.	Vaishnopuram	Pond water	Varanasi	P	A	-
3.	Sidhari	Pond water	Azamgarh	P	A	-
4.	Charbagh	Pond water	Lucknow	A	P	-
5.	Kakadeo	Pond water	Kanpur	A	P	-
6.	Khatima chauraha	Pond water	Sitarganj	P	A	-
7.	Rawatpur	Pond water	Kanpur	P	A	-
8.	Geetanagar	Pond water	Kanpur	P	A	-
9.	Kalyanpur	Pond water	Kanpur	-	-	-
10.	Pratapgarh	Pond water	Pratapgarh	A	P	-

*GC = Green colonies, YC = Yellow colonies, A = Colony absent, P=Colony present

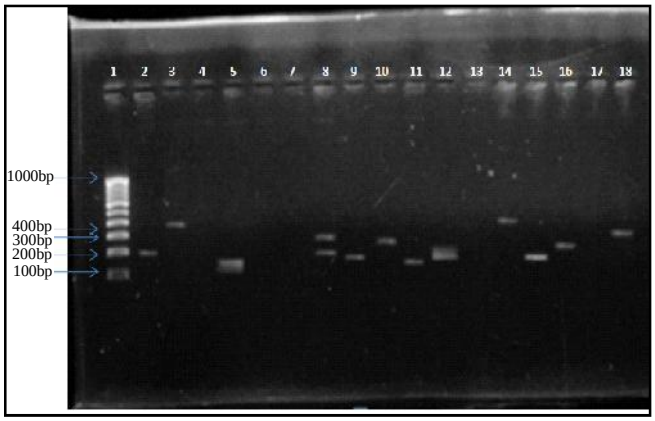

Fig. 1(b) : A 1.2 per cent agarose gel, showing amplified band in lane 2-18, stained in ethidium bromide and visualized under UV light; Lane 1:ladder, Lane 2-18-Amplified PCR bands; Sizes of the amplicons : V_V - 412 bp, V_C - 375 bp, V_P - 96 bp.

etc. However, these methods have failed to distinguish closely related vibrios like *V. harveyi and V. campbellii* (Thompson *et al*., 2004 a and b and Fraser *et al*., 2007). In the present study, 10 pond from U.P. state were targeted to identify prevalence of pathogenic *Vibrio* strains into them. These ponds are used by locals for their regular needs. To identify presence or absence of pathogenic species, water samples were spread onto selective media and further mPCR assay was used to correctly identify the disease-causing bacteria in the present study. Recently similar strategies were followed to successfully identify a number of disease-associated *V. harveyi* strains in a fish hatchery in Malta (Haldar *et al*., 2010). Thus, presence of three *Vibrio* species *i.e*., *V. cholera, V. parahaemolyticus or V. vulnificus* was observed in 6 water samples while the remaining 2 samples showed presence of *V.mimicus* and *V.alginlyticus*.

The antimicrobial assay result of spices showed a maximum activity by aqueous extract liquorice (*Glycyrrhiza glabra*) of 23 ± 0.52 (mm) against *V.vulnificus* followed by mustard seed (*Brassica nigra*) of 22.1 ± 0.60 (mm) against *V. vulnificus* (Table 2). Methanolic and water extract of mustard seed also showed significant activity against *V. cholera* of 20.1 ±0.62 (mm) and 19 ± 0.59 (mm), respectively. The antimicrobial properties of medicinal plants has been explained by the chemical association of active substances; however, the activity of their extracts is not related to their respective dry weights, which can be proven when more effective extracts are considered, e.g. clove extract (95 mg/ml) which had relatively lower activity than guava (122 mg/ml) and garlic (133 mg/ml) extracts and all these extracts showed similar antimicrobial activity patterns.

Spices are heterogeneous collections of a wide variety of volatile and non-volatile staple dietary additives. India with its wide climatic conditions and topographical features naturally possesses wide variety of spices which are being used in the diet. These spices turn an ordinary meal to an extraordinary experience. They contain multiple constituents with antimicrobial activity including phenols, quinones, flavones, tannins, terpenoids, and alkaloids. Some spices and culinary herbs also possess antiadhesive properties, or substances that prevent the adhesion of the microbe to the host tissue, thus, preventing the primary infection point (Pavithra, 2014).

Use of natural products as remedy is being practiced since long time. The practical application and the research works have been greatly neglected in case of

Table 2 : Antimicrobial activity of black cardamom (*Amomum subulatum*), Mustard seed (*Brassica nigra*) and Liquorice (*Glycyrrhiza glabra*) extracts in 3 different solvents against pathogenic *Vibrio* spp.

		Zone of Inhibition (mm)				
Spices used		V.C	V.P	V.V	PC	NC
Black cardamom (*Amomum subulatum*)	Act.	8.10 ± 0.43	12.4 ± 0.68	9.1 ± 0.39	30 ± 1.0	0.0 ± 0.0
	Met.	3.0± 0.0	20 ± 0.7	14.01± 0.68	33 ± 1.0	0.0 ± 0.0
	Wat.	6 ± 0.21	6.5 ± 0.20	7 ± 0.34	29 ± 0.71	0.0 ± 0.0
Mustard seed (*Brassica nigra*)	Act.	0.0 ± 0.0	0.0 ± 0.0	22.1 ± 0.60	29 ± 0.71	0.0 ± 0.0
	Met.	20.1 ±0.62	16 ± 0.52	11.3 ± 0.39	32 ± 0.98	0.0 ± 0.0
	Wat.	19 ± 0.59	17 ± 0.32	15.7 ± 0.41	30 ± 1.0	0.0 ± 0.0
Liquorice (*Glycyrrhiza glabra*)	Act.	16.0 ± 0.32	11.7 ± 0.61	3.4 ± 0.24	24.0 ± 0.72	0.0 ± 0.0
	Met.	12.3 ± 0.42	0.0 ± 0.0	3.4 ± 0.24	24.0 ± 0.72	0.0 ± 0.0
	Wat.	17.33 ± 0.57	11 ± 0.31	23 ± 0.52	28.7 ± 0.68	0.0 ± 0.0

* V_C= V. cholera*;* V_P *=V. parahaemolyticus;* V_V*= V. vulnificus;*

*Act= Acetone extract; Met=Methanol extract; Wat= Water extract; Positive control; NC=Negative control

**Values are expressed as mean ± standard deviation of the three replicates (n=3); Zone of inhibition not include the diameter of the well

formulating remedies against serious life threatening diseases due to the marketed, highly efficient non-natural medical interventions. As far as cholera is concerned, mainly emergence of antibiotic resistant *Vibrio* has rendered the study and application of natural product popular again. Based on the above studies and their discoveries, this particular domain of research can certainly be taken forward and can be directed towards treating contaminated drinking water at storage level or at individual level (Chakraborty, 2015). The aim of this study was to explore such property of plant spices which could prove beneficial for people. As these spices are inexpensive and easily accessible to all, so if they possess antibacterial property against the life threatening water borne microbes, they will be of great use for researchers to find active compounds of these spices.

Conclusion :

The study aimed to use rapid method of identification of *Vibrio* species in pond waters in different regions of Uttar Pradesh. The method used was multiplex PCR which amplifies the TOX gene for determination of the species unlike the traditional methods. Natural compounds of plant extracts may help to explore active compounds which could act as preventive agents against life threatening *Vibrio* species. In this study the crude water, methanolic and acetone extract of three common spices black Cardamom (*Amomum subulatum*), mustard seed (*Brassica nigra*) and liquorice (*Glycyrrhiza glabra*) were investigated. The results indicated that a maximum activity was exhibited by aqueous extract liquorice (*Glycyrrhiza glabra*) of 23 ± 0.52 (mm) against *V. vulnificus* followed by mustard seed (*Brassica nigra*) of 22.1 ± 0.60 (mm) against *V. vulnificus*. The results of the study would help to ascertain the potency of the common spices as potential source of antibacterial agent against *Vibrio* species. Therefore, the extract from these spices can be used for further studies on definitive mechanisms and their active compound which could posses this property.

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