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Bactericidal Efficacy of *Allium sativum* (garlic) Against Multidrug Resistant *Vibrio cholerae* O1 Epidemic Strains

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ABSTRACT

In recent years, emerging trend of antibiotic resistance in *Vibrio cholerae* associated with cholera epidemics is a matter of serious concern for the management of the disease. Indiscriminate use of antibiotics generally results in selection of antibiotic resistant strains. Introduction of newer antibiotics is a challenging task for the researchers as bacteria soon attain resistance. Therefore, identifying natural compounds of medicinal importance for control of cholera would be the best alternative. Garlic (*Allium sativum*) was recognised for many centuries in early Chinese, Egyptian and Indian civilisations as an herbal or traditional medicine. In present study, garlic was selected for screening of antimicrobial efficacy against *V. cholerae*. A total of 55 *V. cholerae* strains isolated from various outbreaks/epidemics were subjected to antimicrobial testing as per CLSI, USA 2010 guidelines. Antimicrobial screening of garlic extract was performed against all the multidrug resistant strains of *V. cholerae***.** The garlic extracts showed antibacterial activity against all the *V. cholerae* strains tested, irrespective of their origin, multidrug resistance and virulence. Antibacterial efficacy of garlic on *V. cholerae* was also evident from *in vivo* study on sealed adult mice model. Thus, the Garlic extract harnesses the potential to control infection of multidrug resistant *V. cholerae,* especially in outbreak like situations in remote and under developed areas where drug supply itself is a challenge.

Keywords: Antimicrobial activity, garlic, multidrug resistance and *V. cholerae*

1. INTRODUCTION

Cholera is a potentially epidemic and life-threatening diarrheal disease that continues to strike many developing countries, where access to safe drinking water and adequate sanitation cannot be assured[1]. Almost every developing country is now facing either a cholera outbreak or the threat of epidemic, which along with serious public health problems severely affects the economy too. The World Health Organisation (WHO) estimates every year there are 1.4 to 4.3 million cases of cholera associated with 28,000 to 1,42,000 deaths worldwide[2]. The disease is endemic in many states of India as per the new definition of endemic cholera devised by WHO Strategic Advisory Group of Experts (SAGE) in 2009[3].

The introduction of oral rehydration suspension (ORS) for cholera treatment by the WHO greatly diminished the disease mortality. Use of effective antibiotics shortens the duration of diarrhea, reduction in the disease severity thereby curbing secondary transmission. While considering cholera management, the emergence of wide-spread antibiotic resistant *V. cholerae* strains presents the greatest challenges for the developing countries[4]. Occurrence of multiple antibiotic resistant *V. cholerae* has been on the rise among outbreak strains[5,6]. Switch in the resistance pattern from one drug to another has also been observed during the management of the outbreaks[7]. Now, most of the *V. cholerae* isolates are becoming resistant to low cost first-line drugs like tetracycline and trimethoprim-sulfamethoxazole[8]. Multidrug resistance in *V. cholerae* is a result of acquisition of genetic elements located within the chromosomal DNA, such as integrons and SXT constin, as vehicles of the transport of genetic determinants responsible for resistance[9-11]. Recently, most of the large cholera outbreaks were associated with *V. cholerae* having multidrug resistance mediated by SXT and integron elements[12-14]. Thus, antimicrobial resistance can increase the outbreak size, duration and case fatality rates. Therefore, alternative treatment approaches like use of antibacterial herbs need to be explored for combating the disease.

The importance of garlic (*Allium sativum*) was recognised for many centuries in early Chinese, Egyptian and Indian civilisations as a herbal or traditional medicine. It is a member of the Liliaceae family and probably one of the earliest known medicinal plants used worldwide to reduce various risk factors[15]. It has sulfur containing compounds like allicin which is considered responsible for its antibacterial properties. Allicin is biosynthesised from alliin by the action of enzyme alliinase. It harbors a thiosulfinate functional group, R-S(O)-

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S-R which is associated with main antimicrobial effect due to its chemical reaction with thiol groups of various microbial enzymes essential for metabolism[16].

Antibacterial properties of garlic extract or allicin have been reported against a number of pathogenic bacteria viz. *E. coli*[17], *V. cholerae*[18, 19], *Pseudomonas aeruginoa*[20], *Salmonella typhimurium*[18], *Streptococcus pneumoniae*[21], *Staphylococcus aureus*[22] and Methicillin resistant *S. aureus* (MRSA)[23,24]. Present study was aimed to check the efficacy of garlic extract against multidrug resistant *V. cholerae* having epidemic potential isolated from different outbreaks in India.

2. MATERIAL AND METHODS

2.1 *V. cholerae* Strains

Total 55 clinical isolates collected from different parts of India (Table 1) were used in this study.

2.2 Antimicrobial Susceptibility Testing

Clinical strains of *V. cholerae* were tested for antimicrobial drug susceptibility by disc diffusion method on Mueller–Hinton agar (MHA) according to the standard procedure[25]. The antibiotic discs (Oxoid limited, UK and Himedia, India) used were: ampicillin (Amp), chloramphenicol (chl), ciprofloxacin (Cip), doxycycline (Dox), erythromycin (Ery), gentamicin (Gen), nalidixic acid (NA), streptomycin (Str), sulfamethoxazole (Sul), tetracycline (TE), trimethoprim (Tri) and the vibrio static agent O/129.

2.3 Preparation of Garlic Extract

Garlic (*Allium Sativum*) extraction was done according to the earlier described methods[21]. The extract was stored in sterilised bottles at - 20 °C. One ml of aqueous extract contained material from 500 mg of garlic. The required dilutions of the garlic extract were prepared in autoclaved double distilled water on the day of experiment. The extract was centrifuged at 5000 g for 15 min; the supernatant was collected and filtered through 0.45 µm membrane filters.

2.4 Determination of Minimum Inhibitory Concentration

MICs of garlic against the randomly selected clinical and environmental *V. cholerae* strains was determined by agar well diffusion and broth micro-dilution using two-fold serial dilutions, as per standard procedure[25].

2.5 Time Kill Assay

Randomly selected *V. cholerae* clinical and environmental strains carrying SXT and integron were subjected to time kill assay[23]. The strains were overnight grown in Mueller Hinton broth (MHB). Bacterial inocula equal to 5 x 10^5 CFU/ml were added to a double strength of MHB (5 ml) containing garlic extract (equivalent to 1 x MIC or 10 x MIC of that particular bacterial strain) and the volume was adjusted to 10 ml by adding sterile double distilled water. The control group which did not receive garlic was run in parallel. A 100 µl of culture broth was harvested at 0 h, 2 h, 4 h, 6 h, and 8 h followed by 10-fold serial dilutions of the inoculums in normal saline and plating on MHA plates. After incubation at 37 °C for 24 h, the colonies were counted. Growth was observed further for 48 h.

2.6 Analysis of Antimicrobial Activity on Thin Layer Chromatographic (TLC) Plate

Garlic extract was run on TLC plates using solvent containing ethyl acetate, methanol and water (81:11:8). *V. cholerae* strains grown to exponential phase were harvested, the inoculum was adjusted to 0.5 McFarland standard and

Table 1. Susceptibility of Multidrug resistant *V. cholerae* clinical isolates (collected from different outbreaks in India) towards aqueous extract of garlic

Year of isolation	Location	No. of strains	Efficacy of garlic	Antibiotic phenotype	Antibiotic genotype		Reference
					SXT	Integron	
2004	Chennai, India	05	+	Na, Str, Sul, Tri, O129	+	+	11
2007	Rayagada, Orissa	05	+	Na, Str, Sul, Tri, O129	+	+	6, 8
2007	Kalahandi, Orissa	05	+	Na, Str, Sul, Tri, O129	+	+	6, 8
2007	Koraput, Orissa	05	+	Na, Str, Sul, Tri, O129	+	+	6, 8
2009	Datia, Morena	05	+	Na, Str, Sul, Tri, O129	+	+	
2009	Hydrabad	05	+	Na, Str, Sul, Tri, O129	+	+	12
2010	Solapur, Maharastra	05	+	Na, Str, Sul, Tri, O129	+	+	
2010	Orissa	05	+	Na, Str, Sul, Tet, Tri, O129	+	+	4
2011	Solapur	05	+	Na, Str, Sul, Tet, Tri, O129	+	+	
2011	Gwalior	05	+	Na, Str, Sul, Tri, O129	+	+	
2012	Yavatmal	05	+	Na, Str, Sul, Tri, O129	+	+	5, 10
	Total	55					

further diluted in molten Mueller Hinton Agar (45 °C - 50 °C) at a final concentration of 5 x 10^5 CFU/ml. The suspension was immediately overlaid on prepared TLC plate and incubated at 37 °C for 24 h.

2.7 LC-MS Analysis of Garlic Extract

For liquid chromatography, the following solvent composition was made for sample introduction: solvent A (0.01M ammonium formate in water) and solvent B (0.01M ammonium formate in methanol). Chromatographic separations were performed using 0 per cent B (0 – 3 min), 0 – 100 per cent B (3 – 30 min) and 100 per cent B (30 – 40 min) at a flow rate of 200 µLmin^{-1}. Column temperature was kept at 25 °C. Samples were injected through a Rheodyne injector (Model 7010) fitted with 2 µL loop.

Mass spectral analyses were performed on Micromass Q-ToF high resolution mass spectrometer equipped with electrospray ionisation (ESI) on Masslynx 4.0 data acquisition system. ESI was used in +ve ionisation mode. Capillary and tube lens voltages were optimised to give the maximum response. Helium was continually flowing into the collision cell at a pressure of 0.1 Pa. (10−3 Torr) during the electrospray ionisation tandem-mass spectrometry (ESI-MS) operation. The ESI-MS data were acquired over the mass range of *m*/*z* 100 amu – 400 amu. The mass spectra were obtained at collision energy of 35 per cent.

2.8 *In vivo* Testing of Garlic Extract in Cholera Model

Balb/c mice (weight 20 g - 24 g) were used in this study. Sealed adult mice (SAM) model was prepared according to the previously described methods with some modifications[26]. In brief, mice were starved overnight, anorectally canal was sealed with nail polish, given 500 µl of 2 per cent $NaHCO_3$ prior to administration of either bacterial inocula (10^9 CFU/ml) or bacteria followed by herbal extract (n = 4 mice/group) after 1 h and sacrificed at 6 h or 24 h. The intestine weight to carcass weight ratios was determined. In this experiment, we used Haitian type of CT producing strains as the strains were associated with a higher mortality in the epidemics. The control group of SAM (n = 4) received only PBS. They were kept in wired-mesh polyacrylic cages and fed with the standard rodent pellet diet and given water *ad libitum*. The animals were housed under standard laboratory environmental conditions for acclimatisation prior to making cholera model and performing the experiments.

3 RESULTS

3.1 Antimicrobial Susceptibility Pattern

The clinical *V. cholerae* isolates exhibited varied phenotypic resistance towards the antibiotics ampicillin, chloramphenicol, aminoglycosides, macrolides, quinolones, fluoroquinolones, sulfomethoxazole, trimethoprim and tetracyclines. The antibiogram of clinical isolates has been presented in Table 1.

3.2 Antibacterial Activity of Garlic Against *V. cholerae* Strains

Garlic aqueous extract presented antibacterial activity against all the clinical isolates tested (Table 1). The garlic extract (500 mg/ml) exhibited the zone of inhibition in the range of 19 mm to 27 mm diameter against clinical isolates (Fig. 1). The MIC in broth dilution ranged from 4 mg/ml - 16 mg/ml of garlic extract for *V. cholerae* ATCC 14033 and randomly selected epidemic strains resistant to important drugs used for cholera treatment viz. tetracycline and ciprofloxacin.

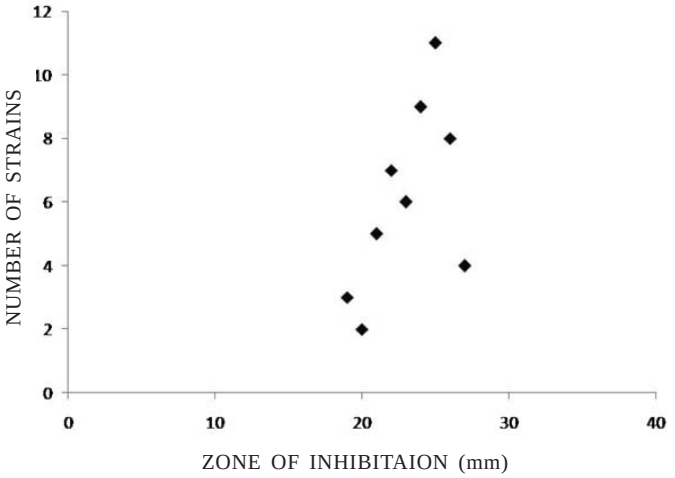

Figure 1. Size of zones of inhibition with garlic aqueous extract (500 mg/mL) against multidrug resistant *V. cholerae.*

3.3 Time Kill Curve

Time kill curves showed that 1X MIC of garlic killed the strains within 6 h, whereas, 10X MIC of garlic greatly reduced the viable count and killed the strains within 4 h (Fig. 2).

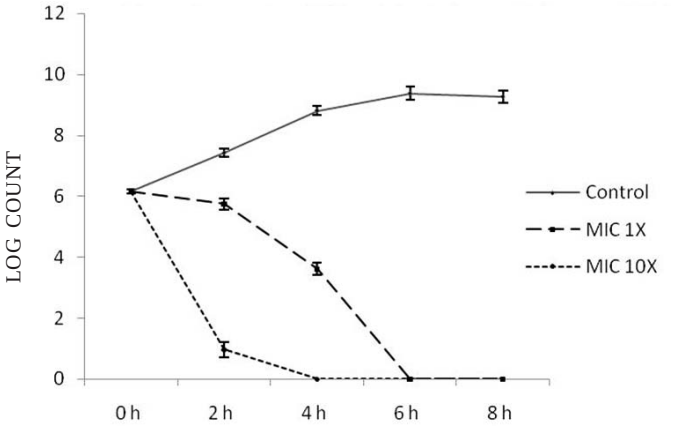

Figure 2. In vitro time dependent bactericidal of effect of garlic extract on multidrug resistant *V. cholerae*. Minimum inhibitory concentration (MIC) of garlic extract was used as MIC-1X and MIC-10X (10 fold) in broth culture.

3.4 Antimicrobial Activity of Garlic Extract on TLC Plate

The zone of clearance was observed on TLC plate

indicating the *V. cholerae* growth inhibition due to presence of active antimicrobial compound at that particular position. A 0.54 R_f value was observed reflecting the zone of inhibition.

3.5 Allicin Peak Identified in LC-MS

Separation of the components of aqueous extracts of garlic was done on a C-18 column. In comparison to a reported retention time, the allicin peak has been tentatively identified (Fig. 3) which is the main component of garlic. On heating the aqueous extract of garlic for about 10 min at boiling water, the allicin peak was reduced significantly as a result of thermal instability of allicin. To confirm the identity of the isolated allicin, the extracts were then analysed by LC-MS.

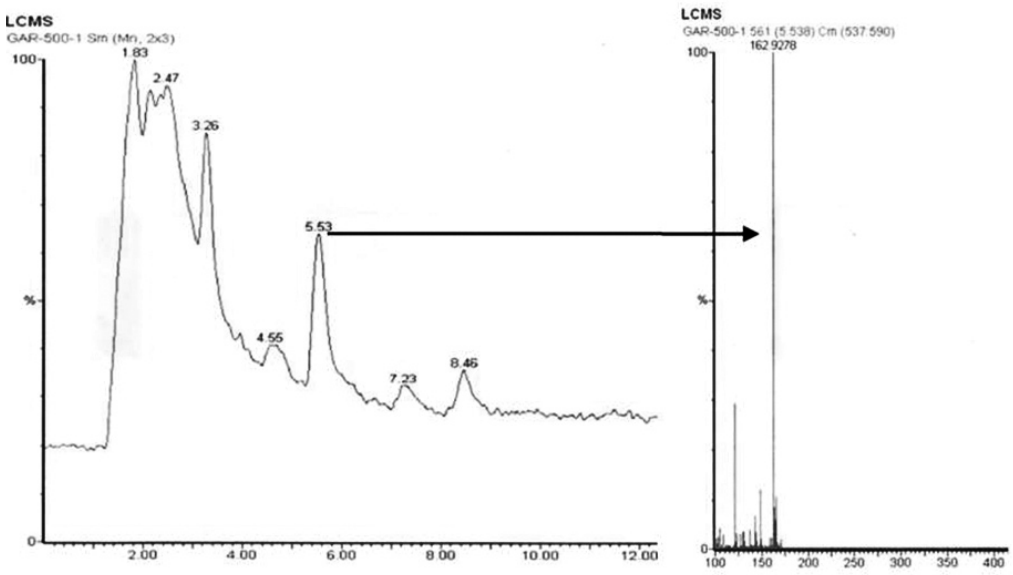

Figure 3. LC-MS profile of *Allium sativum* (garlic) extract.

3.6 In-*vivo* Efficacy of Herbs in Cholera Model

A doze of 1 x 10^9 CFU of the epidemic *V. cholerae* strains (carrying Haitian CT) was able to induce cholera as significant fluid accumulation was observed in SAM (Fig. 4). The group of SAM administered with garlic extract following 1x10^9 CFU of the bacteria showed significant reduction in the fluid accumulation. The FA ratio of treated group was found very near to the control group which received only PBS.

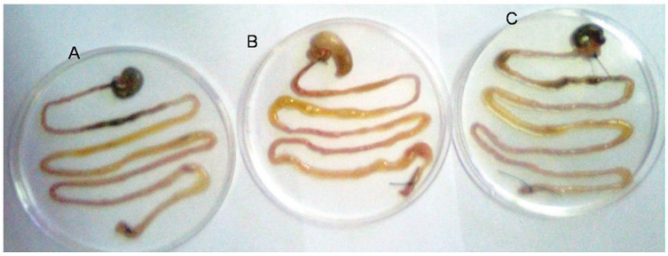

Figure 4. In-*vivo* efficacy of garlic in cholera (SAM) model. A: Mice received buffer only, B: mice received *V. cholerae* and C: Mice received *V. cholerae* followed by administration of garlic extract.

4. DISCUSSION

Multiple antibiotic resistances among *V. cholerae* have emerged as a major problem worldwide. The occurrence of antibiotic-resistant strains of *V. cholerae* is being reported with increasing frequency. In the process, the bacteria are continuously changing their genetic makeup for sustained infectivity[8,13,14,27]. Acquisition of antibiotic resistance genes across genera and species is mediated through horizontal and lateral gene transfer of mobile genetic elements like SXT and integron[10,28].

Inappropriate and inadequate practices like misuse, abuse and over prescription of antibiotics are common in developing countries[29,30]. In developing countries, the antimicrobial therapy is usually started even before the microbiological analysis[5]. Doxycycline and tetracycline are the preferred antibiotics given in severe cases for treatment but in recent year reemergence of tetracycline resistant clones have been documented in India and other parts of the world, raising the question for their use[31]. Till date *V. cholerae* has acquired resistance to all commonly available antibiotics, therefore in outbreak like situation the prescription of effective antibiotic for combating the disease based on the trend of resistance cannot be assured without knowing antibiogram in laboratory conditions, a process that takes at least 2 - 3 days. In that, situation garlic extract can be made easily available for severely diseased persons. Medicinal plants are a rich source of antimicrobial agents and provide a safer and cost effective way of treating bacterial infections. In Ayurvedic and Yunani medicine, herbs have been used to treat many infectious diseases for centuries including the treatment of dysentery and watery diarrhea. Garlic is commonly available and used as dietary supplement all over the world. It is also used to cure abdominal pain and severe diarrhea by Arabian herbalists[32]. The *in vitro* activity of garlic has been reported against diverse enteric pathogen and diarrheagenic bacteria like *E. coli* O157, *Enterobacter cloacae*, *Enterococcus faecalis*, *Citrobacter freundii*, *S. typhi*, *S. dysenteriae*, and *V. cholerae*[17,18,20-22].

The effect of garlic was bactericidal as there was no growth observed on BHI agar till 48 h following 8 h of treatment with garlic (8 mg/ml). The main antimicrobial effect of garlic is due to the presence of diallyl sulphides and allicin is the most abundant sulfoxide molecule in garlic extract responsible for therapeutic potential including bactericidal activity[23,26]. The functional group of allicin react with thiol groups in the active site of various microbial enzymes, e.g. alcohol dehydrogenase, thioredoxin reductase, and RNA polymerase and affect essential microbial metabolism[16]. Allicin is produced upon tissue damage from the non-proteinogenic amino acid alliin (S-allylcysteine sulfoxide) in a reaction that is catalysed by the enzyme alliinase[16]. The activity of alliinase reduces on boiling. Therefore, fresh garlic extracts is prepared

for extraction of optimum active fractions. The European Scientific Cooperative on Phytotherapy recommended garlic to cure upper respiratory tract infections[33]. The antibacterial activity of garlic extract against intestinal bacteria is reported superior than tetracycline[34]. A human trial conducted in different hospitals in China revealed the efficacy of garlic preparation in controlling *Cryptosporidium parvum* diarrhea in AIDS patients with weakened immune system[35]. Our study indicated that aqueous garlic juice can act as broad spectrum antibacterial agent in managing cholera outbreaks. Furthermore, it can be used as nutritional supplement in the affected areas for the prevention of cholera spread.

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Conflict of Interest: None

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