Journal of Rawalpindi Medical College (JRMC); 2016;20(4): 312-316

Original Article

In Vitro Antimicrobial Activity of Garlic (Allium Sativum) Against Clinical Isolates of Vibrio Cholerae

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Abstract

Introduction: To study the in vitro antimicrobial activity of garlic (Allium Sativum) against clinical isolates of Vibrio Cholerae

Methods: Thirty three clinical isolates of V. cholerae were evaluated for their susceptibility to aqueous extract of Pakistani and Chinese garlic. ATCC strain of P. aeruginosa (ATCC 27853) was used as the standard control strain. The cloves were peeled and blended (50 gm) in 90 ml of distilled water in separate blenders. The extracts were sterilized by gamma radiation. Aqueous extracts of Pakistani and Chinese garlic were tested in triplicate. Quantitative analysis of Pakistani and Chinese garlic aqueous extracts was done. As the extracts of both types of Pakistani and Chinese garlic exhibited inhibitory effect against test strain of V. cholerae, aqueous extracts were further evaluated in agar dilution assay to determine MIC against thirty three clinical isolates of V. cholerae and one ATCC reference strain.

Results: The zones of inhibition measured by using agar well diffusion assay were 28.12 mm and 32.14 mm for Pakistani and Chinese garlic aqueous extracts respectively. All the isolates were inhibited at concentration of 8.66 mg/mL by Chinese garlic and at 10 mg/mL by Pakistani garlic aqueous extract.

Conclusion: Natural sources, like garlic extracts , may be an effective alternative, to resistant strains of vibrio cholera.

Key Words: In Vitro , Antimicrobial Activity , Garlic, Allium Sativum, Vibrio Cholerae

Introduction

Cholera is an acute enteric infection which in its most severe form causes acute watery diarrhea that can lead to death within few hours due to dehydration. The first line antibiotics to treat cholera are tetracycline, ampicillin, nalixic acid, furazolidone and co-trimoxazole. Since 1979 their general usefulness has reduced with the emergence of resistance to these antibiotics. Transmission to humans is by food or water. [1,2]

Between 1995 and 2004, the World Health Organization (WHO) reported 100,000 to 300,000 cholera cases. Due to poor surveillance systems and frequent under reporting, WHO estimates that approximately 99% of cholera cases go unreported. WHO estimates that actual case rates approach 3 to 5 million with more than 120,000 of those cases leading to mortality. [3] In 2010, 317 534 cholera cases and 7543 deaths due to cholera were reported globally which represents 52% increase as compared to 2009.[4]

Until 1993, cholera was solely associated with V. cholerae strains of the O1 serogroup. All other strains that were identified as V. cholerae on the basis of biochemical tests, but that did not agglutinate with "O" antiserum were referred to as non-O1 V. cholera. However, in 1993, a new epidemic strain caused cholera like disease in Bangladesh which did not agglutinate with O1 and non O1 antisera. This new strain was given the designation of O 139 Bengal after the area from where it was first isolated.[5] After the recognition of epidemic strain 0139, the term non O1 and non 139 is used to include all other serogroups of V.cholerae except O1 and O139. The non O1 and non O139 are occasionally isolated from cases of diarrhoea, from a variety of extraintestinal infections, wounds, sputum, ear, urine, and cerebrospinal fluid. [6,7] The O1 serogroup has two biotypes, classical and El Tor. On the basis of antigenic factors, O1 serogroup is further differentiated into two major serotypes Ogawa and Inaba. Strains of the Ogawa serotype express the A and B antigens, while Inaba strains express only the A and C antigens. A third serotype, Hikojima, expresses all three antigens but is rare.[8,9]

Antimicrobial therapy decreases the volume of fluid loss, duration of illness, and duration of excretion. The choice of antibiotics for treating cholera is primarily determined by the patterns of bacterial resistance and antibiotic toxicity. Tetracyclines and its derivatives have been the antibiotics of choice for treatment of cholera in adults but not in children due to staining of teeth.[10] Azithromycin, a derivative of erythromycin has been effective for cholera in children. Watery stools stop within two days of giving azithromycin in

more than 70% of patients and V. cholerae is eliminated from stool within two days from the start of treatment in almost 80% of people.[11]

Multiple studies denote that resistance is progressively increasing in developing countries. In 1977, Tanzania was the first country to report multidrug resistant El Tor V. cholerae, followed by several such reports from all over the world.[12] India has reported a sudden upsurge in tetracycline, furazolidone and trimethoprim/sulfamethoxazole resistance among V. cholerae isolates.

Throughout human history, traditional herbal medicine has played a vital in the treatment of various diseases. Although on the bases of historical evidences several plant species had been used to cure different infectious diseases but there are limited scientific publications supporting these evidences.[13]

Garlic is nicknamed as Russian penicillin due to its widespread use as a topical and systemic antimicrobial agent. Its botanical name is Allium sativum, belongs to family Liliaceae, which also includes onions, shallots, leeks, chives and scallions. From centuries garlic have been extensively used in different civilizations to cure infectious diseases.

Material and Methods

Thirty three clinical isolates of V. cholerae were evaluated for their susceptibility to aqueous extract of Pakistani and Chinese garlic. ATCC strain of P. aeruginosa (ATCC 27853) was used as the standard control strain.Antimicrobial susceptibility of the strains to various antibiotics was tested by Kirby-Bauer discs diffusion method according to Clinical and Laboratory Standards Institute (CLSI) protocols.Aqueous extracts of both varieties of Pakistani and Chinese garlic were prepared. The cloves were peeled and blended (50 gm) in 90 ml of distilled water in separate blenders. The extracts were centrifuged at 5000 rpm for twenty minutes and squeezed through sterile muslin cloth to remove insoluble particles. Additional distilled water was added to each extract to make it exactly 100 mL by volume. The extracts were sterilized by gamma radiation at 10 kilo grays. After subtracting the weight of insoluble material from the original weight of cloves, the final concentrations of extracts were determined to be 133 mg/mL. Serial two fold dilutions were prepared for qualitative analysis of extract.Aqueous extracts of Pakistani and Chinese garlic were tested in triplicate by adding 120 µL in each well with the same allocated number on the assay plate with serial two fold diluted concentrations i.e 133, 66.5, 33.25, 16.62, 8.31, 4.15, 2.75, 1.37 mg/mL. An equal quantity of normal saline was filled as a negative control. After application of extracts and controls, the plates were incubated for 18-24 hours at 37˚C. The diameter of the clear zones was measured in mm with digital calipers (Sylac Fowler ultra-call II). Quantitative analysis of Pakistani and Chinese garlic aqueous extracts was done by by agar dilution assay.As the extracts of both types of Pakistani and Chinese garlic exhibited inhibitory effect against test strain of V. cholerae, aqueous extracts were further evaluated in agar dilution assay to determine MIC against thirty three clinical isolates of V. cholerae and one ATCC reference strain. This assay is more sensitive and provides quantitative results which are closer to clinical scenario.Following concentrations of garlic extracts tested were as : 2.0, 3.3, 4.6, 6.0, 7.3, 8.6, 10.0, 11.3, 12.6, 14.0, and 15.33 mg/ml. (for calculations of garlic concentrations from stock solution of garlic extract

Results

With the method of well diffusion and in agar dilution assay, both the garlic extracts exhibited antibacterial activity against the strains of V. cholerae (n=33). However aqueous extract of Chinese garlic exhibited better antimicrobial activity than that of Pakistani garlic against V. cholerae. An examination of the zones of inhibition reveals that the inhibitory activity is linear function of concentration

Table 1: Zones of inhibition of aqueous garlic extracts (A. sativum) against V. cholerae (UHS 1) by agar well diffusion method.

Concentrations of extracts mg/mL	Zone of inhibition (mm)	
	C.G.A.E Mean ± S.D	P.G.A.E Mean ± S.D
133	32.14±0.12	28.12±0.05
66.5	25.71±0.09	24.43±0.11
33.25	23.78±0.07	22.50±0.06
16.62	20.56±0.15	19.56±0.12
8.31	18.17±0.17	16.54±0.13
4.15	14.57±0.11	12.15±0.06
2.75	0 ± 0	0 ± 0
1.37	0 ± 0	0 ± 0
Phenol 6%		34.12±0.13
Normal saline		0 ± 0

C.G.A.E=Chinese garlic aqueous extract, P.G.A.E= Pakistani garlic aqueous extract;S.D = Standard deviation.

Qualitative assay shows that C.G.A.E exhibited 32.14 ± 0.10 mm zone of inhibition at highest concentration of 133 mg/mL and 14.57± 0.13 mm at lowest

concentration of 4.15mg/mL. Whereas P.G.A.E exhibited 28.12± 0.11mm zone of inhibition at concentration of 133 mg/mL and 14.15 ± 0.5 mm at lowest concentration of 4.15 mg/mL. The positive control 6% phenol (w/v) exhibited 34.12 ± 0.62 mm zone against V. cholerae, whereas, negative control normal saline showed no zone of inhibition(Table 1;Fig 1).

Table 2: Minimum inhibitory concentration of Chinese garlic aqueous extract against 33 strains of V. cholerae

MIC of Aqueous extract of Chinese garlic against *V. cholerae*											
Concentration of extract (mg/mL)	2.0	3.33	4.66	6.0	7.33	8.66	10.0	11.33	12.66	14.0	15.3
No of isolates inhibited	0	0	0	7	23	33	33	33	33	33	33
% of isolates inhibited	0%	0%	0%	30%	69%	100%	100%	100%	100%	100%	100%

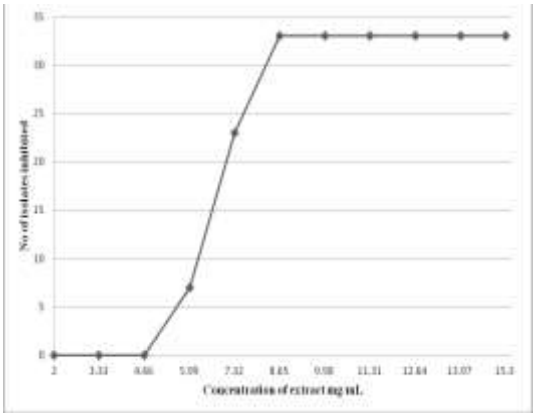


Figure 1: MIC of Chinese garlic extract

Table 3: Minimum inhibitory concentration of aqueous extract of Pakistani garlic against 33 strains of *V. cholerae*

MIC of Aqueous extract of Pakistani garlic against *V. cholerae*											
Concentration of extract (mg/mL)	2.0	3.33	4.6	6.0	7.3	8.66	10.0	11.33	12.66	14.0	15.3
No of isolates inhibited	0	0	0	0	09	22	33	33	33	33	33
% of isolates inhibited	0%	0%	0%	0%	39%	66%	100 %	100%	100%	100 %	100 %

In quantitative analysis of both extracts was done by in agar dilution assay. C.G.A.E inhibited seven out of thirty three clinical isolates of V. cholerae at M.I.C of 6.00 mg/mL, twenty three isolates at M.I.C of 7.33

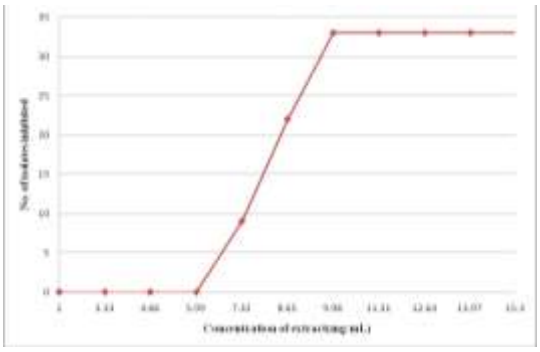


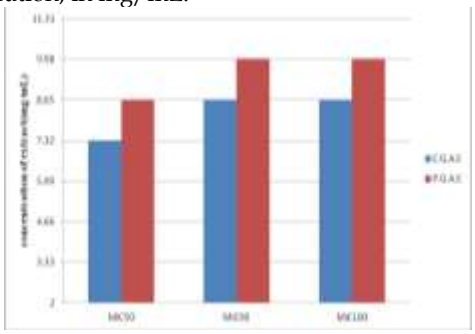
Figure 2: MIC of Pakistani garlic extract

mg/mL, and all thirty three isolates at M.I.C of 8.66 mg/mL (Table 2). P.G.A.E inhibited nine strains at M.I.C of 7.33 mg/mL and all thirty three strains at M.I.C of 10 mg/mL. P. aeruginosa was inhibited at 8.66 mg/mL (Table 3;Fig 2). Statistically significant difference was observed in the mean M.I.C of aqueous extracts of Chinese and Pakistani garlic (table 4;Fig 3).

Table 4: MIC range of aqueous extracts of Pakistani and Chinese garlic against *V. cholerae*

Extract Type	Concentration Range (mg / mL)	MIC_{50}	MIC_{90}	MIC_{100}
C.G.A.E	6.00-8.66	7.33	8.66	8.66
P.G.A.E	7.33-10.0	8.66	10.0	10.0

P.G.A.E = Pakistani garlic aqueous extract C.G.A.E = Chinese garlic aqueous extract;MIC= Minimum inhibitory concentration, in mg/mL.



C.G.A.E= Chinese garlic aqueous extract P.G.A.E= Pakistani garlic aqueous extract

Figure 3: MIC range of aqueous extract of Pakistani and Chinese garlic against vibrio cholerae

Discussion

With the emergence of antibiotic-resistant bacteria it is justified to explore new sources of natural products having antimicrobial activity. The edible plants have been proven to be harmless and economical and garlic

is known to have cure for a variety of bacterial, viral, parasitic and fungal diseases. Various plant extracts have been investigated for their antibacterial activity against V. cholerae including Camellia sinensis (black tea), Citrus limonium (Lemon), Malus sativa (Apple), Cydonia oblonga (membrilo), Persea gratissima (avocado), Lepechinia caulescens, Punica granatum (pomegranate), Allium cepa (onion), Allium ampeloprasum (elephant garlic).[14] In the present study, a statistically significant inhibitory effect of aqueous extract of Pakistani and Chinese garlic against V. cholerae isolates was observed. It is clear from our results that the Chinese garlic has somewhat better antibacterial activity than the Pakistani garlic. The aqueous extract of Chinese garlic inhibited all the thirty three strains of V. cholerae at concentration range from 6.00 to 8.66 mg/mL while that of Pakistani garlic had MIC of 7.33 to 10 mg/mL. This antibacterial effect of garlic extract is probably due to the presence of sulfur containing compounds like thiosulfinates; alliin being the most abundant thiosulfinate found in the intact garlic bulb. When the garlic bulb is crushed, the enzyme alliinase is released from its vacuoles which acts upon the substrate alliin and convert it into allicin.[15] Previous studies with pure allicin have shown that it has inhibitory effects on a wide range of bacteria.[16, 17] A possible explanation of this difference in the MIC of Pakistani and Chinese garlic aqueous extracts may be the variable quantity of active ingredients present in the two varieties of garlic. It has also been reported that the concentration of allicin was twice as much in Chinese garlic compared to garlic cultivated in Europe and India. However, nobody so far has commented on the constituents of Pakistani garlic. Another factor that might contribute to this difference in MIC could be the pH and temperature variations between different steps in the study. Cellini et al., 1996 observed antibacterial activity diminution during room temperature and attributed it to the allicin instability and its transformation with time into more stable components: polysulfides and thiosulphonates.[23]

Different studies have shown that various garlic preparations also exhibited different spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria including species of klebsiella, proteus, escherichia, salmonella, staphylococcus, streptococcus, bacillus, and clostridium.[18] Even acid-fast bacteria such as Mycobacterium tuberculosis are sensitive to garlic.[19] In another in vitro study conducted by Pongsak et al in Thiland, Elephant garlic oil (Allium ampeloprasum) was found to have MIC range from 3.13 to 25 µg/mL against fifteen clinical isolates of V. cholerae. [14] In our study, the MIC values of Pakistani and Chinese garlic aqueous extracts fall in the range of 6 mg/mL to 10 mg/mL. The possible reason for this gross difference in the MIC range may be due to: (a) oil and not the aqueous extract used in the study; oil was extracted directly from the elephant garlic bulb whereas we used 13.3% w/v of aqueous extract and not the oil (b) elephant garlic is not the true garlic as Pakistani and Chinese garlic but a mere variant of leek; the two have different ingredients (c) Pongsak et al used bacterial suspension at 10^4 CFU/mL in BHI broth whereas we carried out our study at 10^8 CFU/mL with the in agar dilution technique. No other comparison can be discussed as there is no evidence of study on this subject.

Our results, however, are consistent with Srinivasan et al.,who investigated the activity of aqueous extract of garlic against various pathogens at 10^8 CFU/mL at different temperature and pH. [20] The MIC of the extract varied from 7 to 21mg/mL for Gram negative bacteria while it was 6 to 11mg/ml for Gram positive bacteria. The Gram positive organism B. subtillis was comparatively more sensitive than other bacteria tested. This may be due to the lipid content of the membranes of the different groups of the microorganisms and the permeability of allicin and other garlic constituents.

Study by Onyeagba et al , showed no activity of aqueous extract has been reported against organisms other than the vibrio. [21] The reason for this nonbacterial activity was because the aqueous extract had been boiled by the researcher meaning thereby that before use boiling destroys antibacterial activity. Tsao et al. reported antibacterial activity of garlic against Staphylococcus aureus, , Streptococcus haematyticus, Streptococcus viridians, Klebsiella pneumoniae, Escherichia coli, Proteus vulgaris, Salmonella enteritidis, Bacillus subtilis, B. pumitus, B. mycoides, B. megaterium, B. thuringiensis, Sarcina lutea, and Serratia marcescens and he had used aqueous extract without boiling as we did in our study. [22]

Cellini et al has demonstrated dose-dependent antimicrobial activity of garlic extract against three different reference strains of H. pylori at concentrations of 2-5 mg per ml. [23] However, heat treatment of the extracts reduced the inhibitory or bactericidal activity against H. pylori. Garlic also exhibited synergistic effects against H. pylori when given along with a proton pump-inhibitor (omeprazole).

Conclusion

Both Chinese and Pakistani garlic aqueous extract have antibacterial activity against V. cholerae. The lack of standardization in different techniques used by various scientist leads to gross variation in results. A standardized methodology is thus needed.

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