ORIGINAL ARTICLE

In-Vitro Synergy of Aqueous Garlic Extract with Ciprofloxacin Against Clinical Isolates of Salmonella Typhi

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[1,3]Conception, planning of research and writing of manuscript, Discussion
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ABSTRACT

Objective: To determine the emerging issue of treatment failure in typhoid by evaluating the activity of a new combination of aqueous garlic extract and ciprofloxacin.

Study Place and Duration: Department of Microbiology, University of Health Sciences Lahore, Pakistan from April 2013-April 2014

Methodology: Synergism of garlic with ciprofloxacin against Typhi was assessed to solve the critical issue of treatment failure with the best known anti-typhoid drugs till now i-e fluoroquinolones. Twenty five ciprofloxacin susceptible Typhi isolates were selected. Aqueous garlic extract (AGE) was prepared and was screened for antibacterial activity by agar well diffusion method which exhibited an inhibition zone of 25.36±1.60 mm against 1 tested isolate.

Results: Minimum Inhibitory concentration 90 (MIC90) of ciprofloxacin and AGE was > 0.25µg/ml and > 20mg/ml respectively as determined by micro broth dilution method. Pseudomonas aeruginosa (ATCC 27853) was used as a reference strain. Synergism of the combination was assessed using microdilution checkerboard titration technique. The fractional inhibitory concentration index (FICIs) for all the isolates were >0.5 <4.

Conclusion: Thus, ciprofloxacin and Aqueous garlic extract (AGE) showed significant antibacterial activity individually against Typhi but Cipro-AGE combination did not prove to be synergistic against Typhi nor against ATCC Pseudomonas aeruginosa 27853.

Keywords: Aqueous garlic extract, Microdilution checkerboard titration technique, Fractional inhibitory concentration index

Introduction

Typhoid fever is a global health concern particularly for the developing world, including Pakistan. Its annual incidence is around 13.5 million cases and the disease led to 190 000 deaths worldwide in 2010[1].

A very high annual incidence of 573 /100 000 of typhoid fever in Indonesia[2] and still higher of 451/100,000 was reported in Pakistan.[3]

Multi drug resistant (MDR) *Salmonella Typhi* exhibiting resistance to all three first line drugs (Ampicillin, Chloramphenicol and Co-trimoxazole) have been reported since 1980s and have led to multiple outbreaks in Southeast Asia, Central Asia, South America and Africa[4,5,6,]

The problem of multi drug resistant (MDR) Typhi was solved by fluoroquinolones and these drugs became the treatment of choice for Typhi. However, the efficacy of fluoroquinolones was questioned due to emergence of Typhi strains resistant to nalidixic acid and with decreased susceptibility to ciprofloxacin (increased MIC).[7,8,9,10]. The rising antimicrobial resistance is because of injudicial use of antimicrobials and is creating a global health crisis[11].

The critical issue of antibiotic resistance and treatment failures led to the need to explore for new antimicrobial agents from various sources like plants. WHO has emphasized the use of traditional medicine in developing countries and estimates that plant extracts or their active constituents are in use as traditional therapies for 80% of the world's population [12, 13].

Since ancient times; garlic (*Allium sativum)* has been used globally to combat bacterial infections. Allicin is the main antibacterial agent of garlic and belongs to class thiosulphinates.[14] Aqueous garlic extract showed excellent activity against drug resistant bacteria and garlic has shown to knock our many enteric pathogens[15,16] The most striking feature of this promising spice is that it can act synergistically with antibiotics. Its synergy with ciprofloxacin was proven against *E.coli* in a rat model in Korea in 2009.[17] In 2010, a study conducted in India reported synergism of garlic with streptomycin against streptomycin- resistant *Staphylococcus aureus* and *E.coli.* [18]

Hence a valuable approach to address the issue of antibiotic resistance among pathogenic bacteria is to formulate new synergistic combination using a commercially available drug with a natural plant having antimicrobial properties. This method would be cost effective and the treatment would be easily available to the community.

Methodology

This quasi experimental study was conducted in the Department of Microbiology, University of Health Sciences Lahore, Pakistan from April 2013-April 2014

Bacterial Isolates: Typhi isolates were obtained from the Department of Microbiology, University of Health Sciences, Lahore and ATCC 27853 *Pseudomonas aeruginosa* was used as reference strain.

They were recovered from microbanks at -70°C, first thawed and then sub-cultured on nutrient agar plates. Working cultures were maintained on nutrient agar slopes at 2-8°C for up to 2 weeks. The Typhi isolates were re-identified by gram staining, biochemical profile using API-20E (Bio-Merieux, France) and confirmed serologically using antisera (Becton Dickinson Difco, USA).

Susceptibility of bacterial isolates to antibiotics: The antimicrobial susceptibility of Typhi isolates to first-line anti-typhoid drugs, nalidixic acid and ciprofloxacin was determined by Kirby-Bauer disc diffusion method in accordance with the Clinical and Laboratory Standards Institute guidelines[19] using commercially available antimicrobial discs (Oxoid, Basingstoke, UK). Twenty five ciprofloxacin susceptible Typhi were selected for the study.

Aqueous Garlic Extract Preparation: Indigenous garlic was procured from local market of Lahore and the aqueous extract was prepared by the method used by Hannan et al. (2012). The peeled garlic cloves were surface sterilized with ethanol for 10 minutes. Ethanol was allowed to evaporate in laminar flow class 1(Pbi Italy) for 10 minutes. One hundred grams of the cloves were homogenized in 200 ml of distilled autoclaved water in a blender. The homogenate was filtered by passing it first through sterile cotton mesh and then through Whattman filter paper 1. Thus crude aqueous garlic extract of 500mg/ml was ready.[20]

Screening for Inhibitory Effect of Aqueous Garlic Extract By Agar Well Diffusion Method: Typhi isolate "S-2" (randomly selected) was screened for the inhibitory effect of the extract. Bacterial suspension equivalent to 0.5 Mc Farland turbidity standard was lawned over the surface of Mueller-Hinton agar (Oxoid). Five wells were cut into the agar with cork borer. Aqueous garlic extract and controls were tested in triplicate by adding 120μl of each in individual wells. 100% extract, 50%, 25%, distilled water (negative control), and 6% phenol (positive control) were run. The diameters of the clear zones were measured in mm with digital caliper after overnight incubation at 37°C (Sylvac)

MIC Determination of AGE: MIC of AGE was determined against 25 Typhi isolates using Mueller Hinton broth with micro broth dilution method [21]. Two fold dilutions with some intervening concentrations were prepared. Dilutions were 320, 160, 80, 50, 40, 30, 20 and 10 mg/ml. 100μl of each dilution was pipetted into wells of microtitre plate along column 1-9. Bacterial suspensions equivalent to 0.5 McFarland were prepared for each isolate (5 x 10^8 CFU/ml) and then diluted 1:100. 100 μl of bacterial suspension was pipetted into the wells along column 1 through 9. A_{12}-H_{12} was dispensed with M H broth and served as sterility control. A_{11}-H_{11} was dispensed with bacterial suspension that served as positive growth control. Plates were sealed with tape and incubated for 24 hrs at 35-37◦C. MIC was read as the lowest concentration of AGE that showed no visible turbidity.

MIC Determination of Ciprofloxacin: Ciprofloxacin base powder was kindly provided by Hilton Pharmaceuticals. MIC of Ciprofloxacin was assessed as for AGE. Two fold dilutions were prepared. The concentrations were 4, 2, 1, 0.5, 0.25, 0.125, 0.064, 0.032 and 0.016μg/ml.

MIC Determination of Age and Ciprofloxacin in Combination: MIC of combination of ciprofloxacin and AGE was determined through Checkerboard titration method. Ciprofloxacin was diluted along X-axis of microtitre plate and AGE was diluted along Y-axis of a microtitre plate. The concentrations of AGE and ciprofloxacin were determined on the basis of MICs obtained against all isolates. The concentrations of ciprofloxacin and AGE ranged from 4μg/ml to 0.032μg/ml and 320mg/ml to 10 mg/ml respectively. MIC was read as the lowest concentration of

ciprofloxacin and AGE combination with no visible turbidity. The interpretation of the checkerboard synergy testing results was determined by the method of Shahnaz et al[23]

The data was entered and analyzed by SPSS 20. Mean ± SD were calculated for zones of inhibition. Fractional inhibitory concentration index of the cipro-AGE was calculated for each isolate to evaluate the synergism between ciprofloxacin and AGE. FICI was calculated by adding FICs for both ciprofloxacin and AGE.

- FICI = FIC Cipro + FIC AGE
- FIC (fractional inhibitory concentrations) of both were calculated as (MIC of Ciprofloxacin or AGE in combination) / (MIC of the antimicrobial agent alone)
- FIC indices were interpreted as synergistic if values were ≤ 0.5, additive or indifferent if > 0.5 to 4.0 and antagonistic if > 4.0[23]

Results

The antibacterial effect of AGE against one isolate of *Typhi* (S-2) is shown in table I. AGE showed significant inhibition zone by agar well diffusion method. At 100% concentration AGE produced an inhibition zone of 25.36±1.60 mm. At 50% concentration, it produced inhibition zone of 22.19±0.65mm and a zone of 14.35±1.20 mm was observed at 25% concentration. Thus, a decline in antibacterial activity was observed with decreasing concentration.

Phenol 6% produced an inhibition zone of 33.50±1.25 mm. Wells containing distilled water (diluent for the extract) did not show any zone of inhibition.

Tables II show the %age of *Typhi* isolates that were inhibited at different concentrations of ciprofloxacin and AGE (when tested alone) respectively. At 0.25μg/ml ciprofloxacin concentration, 80% isolated were inhibited and at 0.5μg/ml, 100% isolates were inhibited.

Table II: Zones of inhibition of S-2 with AGE by agar well diffusion method

Conc. of AGE (aqueous garlic extract)	**Zone of inhibition (mm)**
100%	25.36±1.60
50%	22.19±0.65
25%	14.35±1.20
% age of *Typhi* isolates inhibited at different concentrations of Ciprofloxacin	
MIC of ciprofloxacin ug/ml	**% age of Typhi isolates inhibited**
1	100
0.5	100
0.25	80
0.125	40
0.064	4
0.032	0
% age of *Typhi* isolates inhibited at different concentrations of AGE	
MIC of AGE mg/ml	**% age of isolates Typhi inhibited**
40	100
25	100
20	76
15	4
10	0
5	0

As for as AGE is concerned, 20mg/ml inhibited 76% and 25mg/ml AGE inhibited 100% of the tested *Typhi* isolates.

MIC ranges of Ciprofloxacin and AGE are shown in table III. MIC90 and MIC100 of AGE were found to be >20mg/ml and 25mg/ml respectively. MIC90 and MIC100 of ciprofloxacin were >0.25μg/ml and 0.5μg/ml respectively.

Table I: Checkerboard showing combination of different concentrations of Ciprofloxacin and Aqueous garlic extract

		CIPROFLOXACIN (μg/ml)											
		1	2	3	4	5	6	7	8	9	10	11	12
AGE (mg/ml)	A	1 40	0.5 40	0.25 40	0.125 40	0.064 40	0.032 40	0.016 40	0.008 40	0 40	0	+	-
	B	1 25	0.5 25	0.25 25	0.125 25	0.064 25	0.032 25	0.016 25	0.008 25	0 25	0	C	C
	C	1 20	0.5 20	0.25 20	0.125 20	0.064 20	0.032 20	0.016 20	0.008 20	0 20	0	O	O
	D	1 15	0.5 15	0.25 15	0.125 15	0.064 15	0.032 15	0.016 15	0.008 15	0 15	0	N	N
	E	1 10	0.5 10	0.25 10	0.125 10	0.064 10	0.032 10	0.016 10	0.008 10	0 10	0	T	T
	F	1 5	0.5 5	0.25 5	0.125 5	0.064 5	0.032 5	0.016 5	0.008 5	0 5	0	R	R
	G	1 2.5	0.5 2.5	0.25 2.5	0.125 2.5	0.064 2.5	0.032 2.5	0.016 2.5	0.008 2.5	0 2.5	0	O	O
	H	1 0	0.5 0	0.25 0	0.125 0	0.064 0	0.032 0	0.016 0	0.08 0	0 0	0	L	L

Table III: MIC of ciprofloxacin and AGE against *Typhi* isolates					
Isolates	**Anti microbial agent**	**MIC(** minimum inhibitory concentration)			
		MIC range	**MIC_{50}**	**MIC_{90}**	**MIC_{100}**
Typhi (n=25)	AGE	15-25 mg/ml	> 15 mg/ml	> 20 mg/ml	25 mg/ml
	Ciprofloxacin	0.064-0.5 μg/ml	> 0.125 μg/ml	>0.25 μg/ml	0.5 μg/ml

Table IV is showing MICs of ciprofloxacin and AGE alone and in combination. It also shows FICIs for all isolates. This is evident from these tables that the combination of AGE and ciprofloxacin has no significant effect in reduction of MICs. Some isolates show decreased MICs in combination but for others MIC is the same in combination as when tested alone. FICIs for none of the isolates including *ATCC Pseudomonas aeruginosa* 27853 was ≤ 0.5.

Table V is showing FICIs of ciprofloxacin and AGE combination for the 25 tested isolates of *Typhi.* The data shows that ciprofloxacin and AGE combination did not exhibit synergism against any *Typhi* isolate. Rather it was indifferent against all the 25 isolates and antagonistic against none. Hence, our newly tried combination did not prove to be synergistic against *Typhi* and was indifferent against 100% isolates.

Table V: FIC indices of cipro-AGE combination against *Typhi* isolates	
No. of isolates	**FICI**
0	≤ 0.5
7	1.75
1	1.8
4	2
1	2.25
2	2.8
10	3
0	>4

Susceptibility pattern of *S. typhi* isolates as done by disc diffusion method is shown in Figure I. 100% ciprofloxacin susceptible isolates were selected for the study. Only 24% were susceptible to nalidixic acid, 44% to co-trimoxazole and

Table IV: MICs and FICIs of AGE AND CIPROFLOXACIN					
Isolates	**MIC of Ciprofloxacin alone ug/ml**	**MIC of AGE alone mg/ml**	**MIC of Ciprofloxacin in combination. ug/ml**	**MIC of AGE in combination. mg/ml**	**FICIs**
S-1	0.25	20	0.25	15	**1.75**
S-2	0.25	20	0.50	20	3
S-3	0.25	25	0.25	20	1.8
S-4	0.50	20	0.5	15	1.75
S-5	0.25	20	0.50	20	3
S-6	0.125	25	0.25	25	3
S-7	0.50	20	0.50	15	1.75
S-8	0.25	20	0.50	20	3
S-9	0.25	20	0.50	20	3
S-10	0.25	20	0.25	15	1.75
S-11	0.50	25	0.5	25	2
S-12	0.125	15	0.125	15	2
S-13	0.125	20	0.125	15	1.75
S-14	0.50	20	0.5	20	2
S-15	0.25	20	0.50	20	3
S-16	0.125	25	0.25	20	2.8
S-17	0.125	20	0.125	25	2.25
S-18	0.50	20	0.25	25	1.75
S-19	0.25	25	0.50	25	3
S-20	0.125	20	0.25	20	3
S-21	0.25	20	0.25	20	2
S-22	0.125	20	0.125	15	1.75
S-23	0.125	25	0.25	20	2.8
S-24	0.064	20	0.125	20	3
S-25	0.125	20	0.25	20	3
ATCC 27853*Pseudomonas aeruginosa*	0.50	160	0.50	80	1.5

46% to ampicillin and chloremphenicol. The sensitivity pattern of *Typhi* isolates to various drugs did not show any relation to the results obtained with cipro-AGE combination.

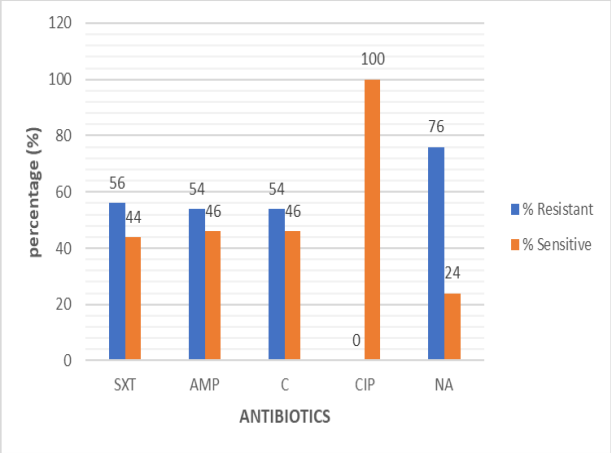

Fig I: Susceptibility pattern of *Typhi* isolates

FICI; fractional inhibitory concentration index
Cipro-AGE; ciprofloxacin and aqueous garlic extract combination
SXT; Co-trimoxazole, AMP; Ampicillin, C;Chloremphenicol ,CIP ;Ciprofloxacin, NA; Nalidixic acid

In present study aqueous garlic extract, when tested alone showed significant activity against Typhi. Our 100% extract had 500mg/ml w/v concentration of garlic in water, and it produced an inhibition zone of 25.36±1.60 mm in agar well diffusion technique.

Discussion

The current study was conducted to address the troublesome problem of fluoroquinolone resistance among Typhi. Keeping in mind the provocative Typhi and synergistic potential of garlic, aqueous garlic extract was combined with ciprofloxacin and the antibacterial potential of the combination was evaluated.

50% concentration exhibited an inhibition zone 22.19±0.65 mm and a zone of 14.35±1.20 mm was produced with 25% concentration. This shows that the antibacterial activity decreased with dilution of extract. MIC range of AGE was 15mg/ml-25mg/ml. These results are consistent with another study at UHS Lahore by Hannan et al[20] where the MIC range of AGE for 50 Typhi isolates including 30 MDR isolates turned out to be 18mg/ml-22 mg/ml by agar dilution method.

In present study, individual MICs of AGE and ciprofloxacin were determined first by micro broth dilution method then MICs of the combination of ciprofloxacin and AGE were determined by checkerboard titration technique and synergism was evaluated by calculating Fractional inhibitory concentration indices. The methodology followed was similar to Nafisa et al.[23] Amoxi-Cassia combination used by Nafisa et al was synergistic against 80% of MDR Typhi. However, in current study though AGE showed significant anti-typhoid activity when tested alone the cipro-AGE combination proved to be indifferent. FICs for all isolates was >0.5 and <4. Hence, the Cipro-AGE combination was neither synergistic nor antagonistic against the tested Typhi isolates as well as against ATCC 27853 *Pseudomonas aeruginosa.* The probable explanation for this is that allicin concentration might have been reduced during aqueous extract preparation. Hence, future studies using pure "Allicin" instead of AGE are suggested. The outcome might be different. Shahnaz et al.[22] conducted a study using pure "Allicin". They proved that allicin and ciprofloxacin showed enhanced antibacterial activity against *Pseudomonas aeruginosa* as compared to ciprofloxacin alone.

The antibacterial potential of garlic owes to its important component "allicin" that principally inhibits bacterial RNA synthesis[24] and ciprofloxacin acts by inhibiting DNA gyrase enzyme thus halting bacterial DNA replication[25]. The difference in the sites of action of the two could favor synergism, as there is a very good example of amoxicillin and gentamicin combination against streptococci. However, our study could not prove synergy of our proposed combination. The results are consistent with some other studies which show either weak or no synergism between nucleic acid inhibitors and plant extracts[25,26] The reason for such behavior of nucleic acid inhibitors should be sorted out and its molecular basis needs to be established.

If this research could prove synergism, the combination could be tried in-vivo and MIC of ciprofloxacin and aqueous garlic extract in combination would be expected to be significantly reduced as compared to the MICs of both alone. This could lead to reduction in dose requirement of ciprofloxacin in typhoid patients and could be a solution to treatment failures with fluoroquinolones. However, the hypothesis couldn't be proved.

Conclusion

Ciprofloxacin and Aqueous garlic extract (AGE) showed significant antibacterial activity individually against S. Typhi but Cipro-AGE combination did not prove to be synergistic against S. Typhi nor against ATCC Pseudomonas aeruginosa 27853.

Limitations of study:

1. Ciprofloxacin was used in pure form; garlic was used in form of crude aqueous extract. There might be a possibility that allicin (active antibacterial component) concentration have been reduced during aqueous extract preparation.
2. Only aqueous extract of garlic was used

Future prospects:

1. Future work with Allicin in pure form instead of extract is proposed so that promising herb, garlic is best utilized and clinical failures of typhoid can be well managed in an affordable way.
2. Ethanolic extract of garlic should be tried.

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