ORIGINAL ARTICLE

ACTIVITY OF SYNERGISTIC COMBINATION AMOXY-CASSIA AGAINST SALMONELLA

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ABSTRACT

The object of this study was to formulate new, cost effective anti-microbial combination for Multi Drug Resistant (MDR) *Salmonella enterica Serover* Typhi (*SEST*) based on the synergistic activity of amoxicillin with the aqueous fruit solution of Cassia fistula (*CFF*), a medicinal plant found in Pakistan which when tested alone have weak antimicrobial activity against blood isolates of MDR *SEST*. MIC of MDR *SEST* for amoxicillin and *CFF* alone was 750µg/ml and 3750µg/ml. The MIC of amoxicillin in combination with *CFF* was 23.4-187.5 µg/ml and 0.37–1.56 µg/ml for MDR and MDS *SEST* respectively. Fractional Inhibitory Concentration Index (FICI) using chequer-board titration suggested synergism for 80% MDR and MDS *SEST* tested, no antagonism observed. Time kill kinetics showed difference > $\log_2$ in CFU of *CFF* having sub-lethal amoxicillin concentrations. This novel combination is named as Amoxy-cassia. In vivo it is found to be non toxic at 1gm/body weight of mice.

Keywords: *Cassia fistula, t*yphoid, beta lactam antibiotics.

INTRODUCTION

Typhoid is a systemic infection caused by *Salmonella enterica Serover Typhi* (*SEST*) (Weinstein *et al.,* 1998) affecting millions of people with 600,000 deaths reported annually worldwide (Merican, 1997) It is endemic in under developed countries and its multi-drug resistant strain is normally present in Indo Pakistan subcontinent, South East Asia and USA. These strains are responsible for several epidemics associated with the consumption of raw food and water contaminated with fecal matter (Chen, 2004; Threlfall, 2002). Chloroamphenicol has been the drug of choice for more than 40 years in different parts of the world where *SEST* remains susceptible to the drug. However, multiple drug resistance to ampicillin, chloroamphenicol, and trimethoprim–sulphamethoxazole has emerged in many countries of Asia and Africa and limited the usefulness of these traditional drugs (Mirza *et al.,* 1996; Jesudason *et al.,* 1996). Though cephalosporin, ceftriaxone, cifrofloxin and cefixime are used against MDR typhoid fever (Bhutta *et al*., 1994) but the communities of patients who suffer from typhoid are poor and these drugs are expensive so they usually do not complete the full course of drug. This situation has led to the development of resistant strains against these drugs too (Van *et al*., 2005; Escribano *et al*., 2004). There are two basic approaches to cure and control the infection caused by the MDR strains of bacteria. First is to explore herbs and higher plants for the isolation of active components that can help to prevent the spread of infection. In this regard WHO is also encouraging its member countries to develop potentially new drugs for chemotherapy of infectious disease from plants used in folklore medicine (Dimayuga *et al*., 1996) but it requires years of research and a lot of capital before a product can be marketed. A second approach is to formulate new synergistic combinations using two commercially available antibiotics, or to combine an antibiotic with a natural plant product having antimicrobial properties. The latter method is cost effective and less time consuming. Many groups of microbiologist are working to formulate different synergistic combination against MDR organisms (Veloira *et al*., 2000; Dawis *et al*., 2003; and Mandal *et al*., 2003).

This study was designed to formulate new, cost effective anti-microbial combination for Multi Drug Resistant *SEST* based on the synergistic activity of amoxicillin with the aqueous fruit solution of *Cassia fistula* (*CFF*) which when tested alone have weak antimicrobial activity against MDR *SEST*. *C fistula* (leguminacea) is commonly known as amaltas. It is also called golden shower because

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of its gorgeous long clusters of bright yellow flowers. Amaltas is an important plant of the ayuverdic system of medicine. Its leaves and bark used to treat roundworm infections, facial paralysis and rheumatism (Jayaweera, 1981). Raw pulp from the pod is used as laxative, employed in treatment of diabetes and its ether extract reported to have a broad – spectrum antibacterial activity (Nadkani, 1979; Bhakata, 1998; Bhavan, 1992). This is the first report of water solution of *C fistula* showing synergistic activity with standard antibiotic amoxicillin against MDR *SEST*. Amoxicillin, an analog of ampicillin is a beta lactam antibiotic, derived from the basic penicillin that has been widely used due to its previously known broad-spectrum antibacterial activity. Currently most microbes have become resistant to it either by producing beta lactamase, an enzyme that inactivates amoxicillin or by producing Modified Penicillin – Binding Protein (PBP) (Maiti *et al*., 1998).

MATERIALS AND METHODS

Bacterial isolates

Forty-eight isolates each of MDR and MDS *SEST* were isolated from the blood samples of patients at the Liaquat National Hospital, Karachi, Pakistan. All isolates identified by standard biochemical reactions (Holt *et al.*, 1994). They were stored at -70 ^{0} C. Fresh subcultures used for each experiment.

Antimicrobial agents

The antibiotic used included amoxicillin as sodium salt from Beecham Pakistan (Pvt.) Ltd. Sulfamethoxazole from Squibb Pakistan (Pvt.) Ltd. and chloroamphenicol powder by Parke Davis and Company Karachi.

Disc diffusion

Bacterial susceptibility to 15 µg/ml chloroamphinicol, 30 µg/ml sulfamethoxazole, 15 µg/ml amoxicillin, 150 µg/ml *CFF* alone and in combination 150 µg/ml *CFF* and 15 µg/ml Amoxicillin was determined in accordance with NCCLS guidelines (1997) using Mueller–Hinton agar (Becton Dickinson, Baltimore, MD, USA).

Plant collection and authentication

Fruit of *C. fistula* were plucked from the trees growing at Department of Microbiology, Karachi University, Karachi, Pakistan in the month of July. Taxonomist Mr Jan Alam authenticated the plant. A voucher specimen bearing general herbarium number 68508 deposited in herbarium of Karachi University.

Preparation of the stock solutions

I. *C fistul*a: The fruits were first shade dried, ground in to powder form, *CFF* powder was dissolved in 100 ml of the sterile distilled water to form 3% solution (w/v), boiled for two minutes with continuous stirring, cooled, and left undisturbed for two minutes and the procedure was repeated twice. Extract was filtered under sterile conditions and the filtrate stored at 0 ^{o} C.

II. *Amoxicillin:* Amoxicillin dissolved in sterile distilled water to form 0.3% solution (w/v).

III. *Amoxy-cassia*: It is the water solution containing 3% (w/v) *CFF* powder and 0.3% (w/v) amoxicillin.

MIC determination

Micro broth dilution method was used and experiment performed in accordance with NCCLS protocols. Briefly, overnight bacterial cultures were used to prepare 0.5 McFarland standard suspensions, which were further diluted 1:5 (~$2x10^6$ CFU/ ml) in action - adjusted MHB (BBL, Cockeysville, MD, USA). Two fold dilution of amoxicillin and *C fistula* was prepared keeping the final volume constant to 0.2ml. Cultures were incubated for 24 h at 37^{o}C. The MIC expressed as the lowest antimicrobial concentration inhibiting the growth for 24h. Viable bacterial counts (CFU/ ml) were determined by standard plating on nutrient agar (Visalli *et al*., 1998).

Chequer board synergy testing

The micro dilution method as described by Visalli *et al*., 1998 served to determine the activity of amoxicillin in combination with *CFF*. They were tested at five different concentrations that ranged 0.195–3.12 mg/ml for *CFF* and 0.039–12.5 µg/ml for amoxicillin against MDS *SEST*. For MDR strain the concentration of *CFF* and amoxicillin were 234–3750 µg/ml and 234–3750 µg/ml respectively. Fractional Inhibitory (FIC) index was calculated as the summation of FICI = $FIC_{amoxicillin}$ + $FIC_{C\ fistula}$. A mean FICI was calculated practical to a commonly utilized definition of synergy and classified either as synergistic (< 0.5) or antagonistic (0.2> 4).

Experimental animals

Six weeks old BALB/c mice of either sex weighing 20–25 gm were obtained from the animal house of HEJ Research Institute of Chemistry. The animals were maintained on the standard pellet diet and water ad libitum during the entire trial period.

Determination of LD50

Animals divided into 4 set. Each set consisted of twenty four animals. Animals in set A were intraperetoneally injected with 1000,500, 250 and 125 mg of aqueous fruit extract / Kg body weight of mice. Set B was given 100, 50, 25 and 12.5 mg of amoxicillin/kg body weight of the mice. Set C given a combination of fruit extract and amoxicillin in the concentration of 1000:100, 500:50, 250:25 and 125:12.5 mg/body weight of the mice. Set D animals injected with saline. The treatment repeated every other day for 10 days. Animals kept under observation for four weeks (Kuehne RW, 1983).

RESULTS

The result of disc diffusion assays showed zone of inhibition measuring 20 mm around the disc impregnated with combination of *CFF* and amoxicillin while no zone was observed around the disc with amoxicillin alone, *C fistula* alone, chloroamphinicol and trimethoprim-sulfamethoxazole (co-trimoxazole).

MIC determination

MIC of the amoxicillin for MDR and MDS *SEST* strains was 750 µg/ml and 1.5 – 12 µg/ml respectively and MIC of *CFF* for both the strains ranged 3750 – 7500 µg/ml and 468 – 3750 µg/ml respectively (table 1).

Chequerboard method for synergy studies

MIC of amoxicillin in combination with *CFF* for MDR and MDS strains of *SEST* was 23.4–187.5 µg/ml and 0.37–1.56 µg/ml respectively whereas MIC value of *C fistula* was 3750–7500 µg/ml and 468–1875 µg/ml respectively. FICI value calculated to be 0.094–0.75 and 0.156–1.5 respectively (table 1).

Time kill kinetics

The time kill kinetic graph showed (fig. 1) that combination (Amoxy – cassia) treated MDR *SEST* strains remained at lag phase for one hour. During 1 - 4 hr they started multiplying at much slower rate as compared to MDR strains growing in the presence of amoxicillin. During the next 4-5 hours the bacterial strains multiplied at faster rate and at the end of fifth hour they started dying out rapidly. In case of control without *CFF*, amoxicillin and their combination MDR *SEST* strains went into log phase much earlier as compared to treated bacteria. The difference in CFU in case of bacteria growing in presence of combination having just sub-lethal concentration of amoxicillin and *CFF* respectively remains > log 2.

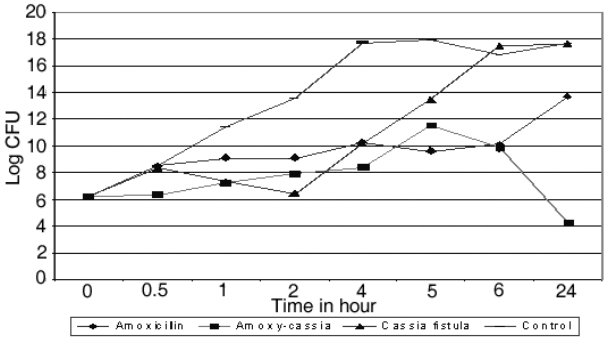

Fig. 1: Time-kill kinetics for an isolate of Multidrug resistant *Salmonella enterica serovar typhi* against Amoxicillin (at MIC >750µg/ml), *Cassia fistula* (at MIC >7500µg/ml), Amoxycassia (Amoxicillin + Cassia fistula at 1:10 dilution of their respective MICs) and control (untreated organism) were tested at different time intervals to confirm the synergistic effect of Amoxycassia. Synergistic efficacy of Amoxycassia was observed throughout the growth cycle. Log_2 decrease in the CFU was seen from 30 minutes to 240 minutes of incubation as compare to Amoxicillin alone however, rapid decrease in CFU was observed from 300 minutes to 360 minutes. Results are given as average $\pm$ standard deviation for three experiments. P values in a two tailed student t test for log CFU/ml of Amoxycassia is less than control (untreated organism) at 360 minutes (P= 0.059).

Statistical analysis

Results are given as average + standard deviation for three experiments. P values in a two tailed student t test for log CFU/ml of Amoxy-cassia is less than control (untreated organism) at 360 minutes (P= 0.059).

In vivo toxicity

BALB/c mice were treated with different concentrations of *CFF* alone and in combination with amoxicillin. The observation recorded showed that treatment up to the concentration of 1 - gm/ Kg of the body weight of BALB/c mice did not reveal any significant affect on the weight of the mice, nor did it bring about any changes in their fur texture. Rather it was interesting to note that treated mice became more active than control mice injected with saline (Kuehne, 1983).

DISCUSSION

The emergence of antimicrobial-resistant bacterial pathogens has become a major public health concern. Indiscriminate use of antibiotics for treatment of disease and as growth promoters in domestic livestock has potentially led to widespread dissemination of Antimicrobial-resistant bacteria (Threlfall, 2002). Several reports indicated isolation of *SEST* that are resistant to chloramphenicol, ampicillin and cotrimoxazole in different parts of the world (Karinki, 2004). Currently the drug of choice for children is cefixime in case of serious infection by MDR strains ciprofloxin is used. Treatment of typhoid fever in male and non pregnant and non lactating females is ceftriaxone, a third generation cephalosporin and ciprofloxin, a flouroquinolone is used but recent studies carried out in different parts of the world indicated a gradual increase in quinolones resistance mutants of *SEST* (Jamil *et al.*, 2004, Abraham *et al.*, 2004).

Vaccines are available to prevent typhoid fever but they are generally reserved for people traveling to underdeveloped countries where significant exposure to *SEST* may occur. But at times the protection offered by the vaccine is partial and may not be effective when visiting areas where exposure is intense. Amoxicillin being the most commonly used broad – spectrum antibiotic with good safety profile to cure typhoid fever.

Its résistance to amoxicillin has been a major cause of concern that is a beta lactam antibiotic belonging to the class of penicillin. Most of the bacteria have developed resistance to amoxicillin by producing beta lactamase, an enzyme that degrades beta lactam ring. The production of beta lactamase is frequently plasmid encoded facilitating the easy transfer of resistance to other strains (Shanahan, 1998). Some of the MDR strains of *SEST* synthesize a modified Penicillin Binding Protein (PBP) that lacks amoxicillin binding site. This prohibits the interaction of amoxicillin with bacterial strain. In order to enhance the antimicrobial activity of amoxicillin, it is combined with the beta lactamase inhibitor clavulanate (Finlay, 2003) this combination is commercially available as augmentin; unfortunately most of the Salmonella strains have developed resistance to it. Another popular method used to enhance activity of any antibiotic that has lost its efficacy is to combine it with another antimicrobial compound, which targets a different biosynthetic pathway. This is true for the synergistic combination of sulfamethoazole and trimethoprim that attacks two different paths of folic acid cycle.

We combined amoxicillin with water extract of fruit of *C fistula* rather than combining it with another antibiotic having a different mode of action. The prime reason for this selection is that higher plants are the rich source of antimicrobial and bioactive substances and traditional

Table 1: MIC of amoxicillin and *C fistula* fruit Alone and in combination (Amoxy-cassia) for MDR and MDS *S. enterica* serover Typhi.

MDR *S. enterica* serover Typhi	MIC AMX alone µg/mL	MIC AMX combination µg/mL	MDR *S. enterica* serover Typhi	MIC *CFF* alone µg/mL	MIC *CFF* combination µg/mL
35%	≥1500	48	18%	7500	937
30%	≥1500	24	18%	7500	1875
20%	≥1500	96	32%	3750	468
08%	≥1500	192	32%	15000	1875
07%	≥1500	384	-	-	-
MDS *S. enterica* serover Typhi					
12%	1.56	0.39	48%	3120	390
12%	1.56	1.56	10%	3120	7780
24%	3.125	0.39	10%	1560	390
12%	3.125	0.78	06%	1560	780
06%	3.125	1.56	12%	390	390
06%	6.25	1.56	08%	3120	1560
08%	12.5	0.39	-	-	-

CFF Water solution of *C. fistula* fruit

Table 2: Fractional inhibitory concentration index (FICI) of amoxicillin and *CFF* for multi-drug resistant (MDR) and multi drug sensitive (MDS) *S. enterica* serover Typhi strains

MDR *S. enterica* serover Typhi	FIC	MDS *S. enterica* serover Typhi	FIC
12%	0.18	8%	0.185
55%	0.25	18%	0.25
33%	0.5	48%	0.375
		14%	0.5
		12%	1.125

medicinal plants are basic part of the health care system in developing countries (Dimayuga, 1996) moreover twenty percent of the available allopathic drug has an active principal obtained from higher plants (Wijesekera, 1991). The therapeutic value of plants belonging to the genus of Cassia has been recognized by several system of medicine. Different parts of *C. fistula* are reported to have anti-bacterial (Perumal, 1998), insecticidal, anti-viral and anti-fungal activities. Mixtures of *CFF* pulp, watery extract of bark and leaves when applied locally help to cure various types of dermatides (Misra and Sinha, 1981). The ether extract of the pulp exhibited broad-spectrum antibacterial activity and found to be more potent than chloramphenicol (Nadkani, 1979; Bhakara, 1998; Bhavan 1992). In present study water solution of fruit was preferred over organic extract as both amoxicillin and *C fistula* are consumed in oral dosage form. Beside it was convenient, cost effective and less time consuming to prepare it. This is the first report on water solution exhibiting synergistic activity with amoxicillin for *SEST*. In an attempt to formulate a new synergistic antimicrobial for MDR *SEST* we combined *CFF* and amoxicillin at the ratio of 10:1 By time-kill kinetics, amoxicillin and *C fistula* exhibited synergy at sub inhibitory concentration as shown in fig 1. This combination was named as Amoxy-cassia. The exact mechanism of synergy is currently unknown but several hypotheses can be put forward to explain its mechanism of action. Firstly, if *CFF* disrupts Lipopolysaccharide, an outer layer of *SEST* it may help in restoring a porins channel thus facilitating the flow of amoxicillin to target sites. Secondly, it may cause negative effects on the efflux mechanism and let the sufficient concentration of amoxicilin to remain in bacterium thus helping in its antimicrobial activity (Stermitz, 2000; Gibbon and Udo EE, 2000, Van *et al*. 2000) Thirdly, *CFF* may be inhibiting protein synthesis as while acting alone it inhibits the growth of MDR and MDS strains of salmonella tested (Koneman, 1997). Currently many antibiotics and natural plant products are available to treat typhoid fever (Rani and Khullar 2004) but it is observed that a bacterium conveniently develops resistance to the single antibiotic preparation. Amoxy-cassia being a synergistic combination, the probability of development of resistant mutants against it will need a long span of time. Almost all of the currently available antibiotics exhibit side effects but our in-vivo study revealed that *CFF* alone and in combination is found to be non toxic in BALB/c mice at concentration of 1gm/Kg of the body weight. Its popular use to treat black water fever; blood poisoning, anthrax and dysentery in South Rhodesia further confirm its non toxic effects in humans (Jaweera, 1981).

CONCLUSION

Amoxy-cassia has the potential to emerge as a new source of treating MDR typhoid if it is found non-toxic in human clinical trials However There is a further need to carry out *in-vitro* and *in-vivo* studies of the combination of *CFF* and amoxicillin to study its mode of action.

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