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Full Length Research Paper

Cardiospermum grandiflorum leaf extract potentiates amoxicillin activity on *Staphylococcus aureus*

Petra O. Nnamani, Franklin C. Kenechukwu* and Wilfred N. Oguamanam

Drug Delivery Research Unit, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka 410001, Enugu State, Nigeria.

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Concurrent administration of orthodox and herbal antibacterial agents could help overcome the tendency with which the former is inactivated by bacterially-produced endogenous enzymes. This study was designed to evaluate the antimicrobial interaction between the ethanol extract of *Cardiospermum grandiflorum* leaf (CGL) which is used in a remote village in Nsukka, Nigeria as a bathing sponge for treatment of skin infections and amoxicillin (AMX), an extended-spectrum but penicillinase-susceptible penicillin. The antimicrobial interaction between these two agents was evaluated by modification of the checkerboard technique using *Staphylococcus aureus* (a penicillinase-producing Gram-positive bacterium)and *Bacillus subtilis* (a non penicillinase-producing Gram-positive bacterium) as the test organisms. The MIC of the ethanolic extract against *S. aureus* and *B. subtilis* was respectively, 25.0 ± 0.1 and 50.0 ± 0.5 mg/ml while the MICs of amoxicillin were 0.05 ± 0.01 and 0.025 ± 0.002 mg/ml against *B. subtilis* and S. *aureus,* respectively. The effect of combination of the ethanol extract of *C. grandiflorum* leaf with amoxicillin was dependent on both the ratio of combination and the test organism employed for the evaluation. Overall, the combined antimicrobial effect was predominantly synergistic against *S. aureus*.

Key words: *Cardiospermum grandiflorum* leaf, antibacterial interaction, checkerboard technique, *Bacillus subtilis*, *Staphylococcus aureus*, amoxicillin.

INTRODUCTION

Recently, scientific interest in medicinal plants have burgeoned due to the increased efficiency of plant derived drugs and less side effects of the latter compared to modern medicines, continuing emergence of drug resistant organisms and adaptations by microbial pathogens to commonly used antimicrobials (Nair and Chanda, 2006; Parek et al., 2006; Audu et al., 2004; Adeniyi et al., 2005; Akinpelu and Onakoya, 2006; Nkere and Iroegbu, 2005). Ballon vine (*Cardiospermum grandiflorum* Fam. Sapindaceae), a vigorous, vine-like climber (twinner), pubescent or nearly glabrous annual or perennial plant has slender branches, ternately compound, membraneous, depressed, pyriform capsule leaves wrangled at the angles with black seeds having large white shaped aril (Aluka, 2008). Various parts of the plant such as the leaves, roots and seeds have been widely used in traditional medicines for curing various human ailments including treatment of arthritis, amenorrhea, lumbago, neuropathy and rheumatism, stiffness of limbs and snake bite, nervous disorders and piles, diarrhoea, diabetes, convulsion and bacterial infections (Aluka, 2008; Banso, 2007).

Amoxicillin, a synthetic extended-spectrum penicillin is known to decrease the stability of the cell wall by inhibiting both the transpeptidase and the D-alanine carboxypeptidase enzymes (Chambers, 2004). *Staphylococcus aureus* is a normal flora of the skin and is also implicated in some opportunistic infections of the

*Corresponding author. E-mail: chimafrankduff@yahoo.com, frankline.kenechukwu@unn.edu.ng. Tel: +234 8038362638.

Abbreviations: CGL, *Cardiospermum grandiflorum* leaf ethanolic extract; **AMX,** Amoxicillin; **FIC_{AMX},** fractional inhibitory concentration of amoxicillin; **FIC_{CGL},** fractional inhibitory concentration of *C. grandiflorum* leaf ethanolic extract; **Syn,** synergism; **Add,** additivity; **Ind,** indiffernce; **Ant,** antagonism.

Figure 1. Picture of *C. grandiflorum* leaf.

skin (Nair and Chanda, 2006; Parek et al., 2006; Nkere and Iroegbu, 2005). Amoxicillin is inactivated by penicillinases including those produced by *S. aureus* (Chambers, 2004).

The interest in the present study was spurred by our observation, over the years, that a large number of patients who had some skin infections (characterized by massive dry skin with somewhat large patches of spreading scaly non-itching skin) on several occasions in a remote village in Nsukka L.G.A. of Enugu State, Nigeria were successfully treated by traditional medicine practitioners with the fresh leaves of *C. grandiflorum* as bathing sponge. These patients resorted to traditional medicines after unsuccessful attempts to treat the skin infection with conventional antibiotics due to reoccurrence of the infection. To authenticate this folkloric use, the plant was taken to a botanist for proper identification, hence the conception of the work.

In rational drug therapy, the concurrent administration of two or more antimicrobial agents is often essential and sometimes mandatory in order to achieve the desired therapeutic aim to treat specific infections and co-existing diseases as well as prevent the emergence of resistant micro-organisms (Nnamani et al., 2005). Drug interaction may result in synergistic, antagonistic, indifferent or additive effects (Esimone et al., 2002). It is highly expedient that the *in vitro* interaction of combination of antimicrobials be evaluated using suitable test microorganisms before such combinations are clinically used. Although a study has been carried out that established the antimicrobial activities of crude ethanol extract from leaf of *C. grandiflorum* (Banso, 2007), to the best of our knowledge there is paucity of information on the antibacterial properties of crude ethanol extract from leaf of *C. grandiflorum* in combination with conventional antibiotics.

In this paper therefore, we report on the antibacterial interaction of crude ethanol extract of *C. grandiflorum* leaf with amoxicillin, an extended-spectrum but penicillinase-susceptible penicillin.

MATERIALS AND METHODS

Reagents

Analytical grades of ethanol 99% (Fluka, Germany) and dimethylsulphoxide, DMSO (Merk, Germany) were used for extraction and dilution respectively of the *C. grandiflorum* leaf extract. Distilled water was collected from an all-glass still. Nutrient agar (Fluka, Germany) was used as medium for the study. Amoxicillin pure powder (Afrab-Chem. Ltd., Nigeria) was used as synthetic antibiotic. Laboratory isolates of *S. aureus* and *Bacillus subtilis* were obtained from stock cultures in the Pharmaceutical Microbiology laboratory, Department of Pharmaceutics, University of Nigeria, Nsukka.

Collection and identification of plant material

Fresh leaves of the *C. grandiflorum* were obtained in June, 2009 from Nkalagu-Obukpa in the Nsukka locality (Figure 1). Authentication of the leaves was done by Mr. A. O. Ozioko of the Bioresources Development and Conservation Programme Center (BDCP), Nsukka, Enugu State, Nigeria and a voucher specimen (PC98132) is preserved in the Pharmacognosy Herbarium, University of Nigeria, Nsukka.

Preparation of the *Cardiospermum grandiflorum* leaf ethanol extract

The *C. grandiflorum* leaves were air dried under shade for two consecutive days and then pulverized using electric blender at the Soil Science Department of the University of Nigeria, Nsukka. Approximately 250 g of the fine powder was extracted with one litre of 99% ethanol by the cold maceration method for 24 h. The extract

was further filtered, allowed to evaporate to a semi-solid residue and stored at 25°C until required for use.

Preparation of culture media

The growth medium employed was nutrient agar and it was prepared using the methods specified in the Oxoid manual (Oxoid, England). The stock microbial cultures were maintained on nutrient agar slants at 4°C. In order to activate these cultures, subcultures were freshly prepared and incubated at 37°C for 18 to 24 h before use. Standard suspensions of each test microorganisms were made by transferring a colony from the subculture containing approximately 10^9 colony forming unit per ml (cfu/ml) of the organisms into 5 ml of sterile distilled water, and adjusting the volume to obtain a cell population of approximately 10^6 cfu/ml. A volume of 0.1 ml of such suspensions was used as inoculum in all the tests.

Preliminary antimicrobial screening

Preliminary antimicrobial screening of the *C. grandiflorum* leaf extract was carried out using the cup-plate agar diffusion method (Hassan et al., 2003). This method depends on the diffusion of antibiotics from holes on the surface of the microbial seeded agar. Molten nutrient agar (20 ml) was inoculated with 0.1 ml of *S. aureus* broth culture. It was mixed thoroughly, poured into sterile Petri dishes and rotated for even distribution of the organism. The agar plates were allowed to set and a sterile cork borer (8 mm diameter) was used to bore six holes in the seeded agar medium. Two drops of each of the two-fold dilution of the extract in DMSO (100, 50, 25, 12.5, 6.25 µm, 3.125 mg/ml) was added into each labeled hole using a sterile pipette.

The plates were allowed to stand at room temperature for 15 min to enable the samples to diffuse into the medium before incubating at 37°C for 24 h. The experiment was repeated for *B. subtilis*. Three replicate tests were performed in each case. Growth was examined after incubation and the diameter of each inhibition zone was measured and the average determined. A control experiment was also set up against each test organism using DMSO as a control diluent. The whole experiment was similarly repeated for 4 mg/ml of amoxicillin using sterile distilled water as the solvent for dilution.

Determination of the minimum inhibitory concentration (MIC)

The MIC of the *C. grandiflorum* leaf extract was obtained using the agar dilution technique (Ofokansi et al., 2008). A stock solution of the extract (100 mg/ml) was prepared by dissolving 200 mg of the extract in 2 ml of 50% DMSO (that is, one part of DMSO in one part of water). Then two-fold serial dilutions were made with sterile distilled water to obtain concentrations between 50 and 3.125 mg/ml. A volume of each of the concentrations equal to 0.5 ml was transferred into an agar plate and made up to 20 ml with molten agar and then allowed to set. The surface of the agar was then dried and streaked with isolates.

An over-night (24 h) broth culture was used for this experiment. The same procedure was repeated with amoxicillin but in this case a stock solution of 4 mg/ml was prepared and the final concentrations obtained in agar plates ranged from 0.4 to 0.025 mg/ml. Control plate having 5 ml of 50% DMSO in 15 ml of molten agar was prepared for *C. grandiflorum*. The plates were then incubated at 37°C for 24 h. The MIC was taken to be the lowest concentration which showed no visible growth of each of the test isolate on the agar surface. The experiment in each case was carried out in three replicates.

Evaluation of the interaction between *Cardiospermum grandiflorum* leaf extract and amoxicillin

Stock solutions of *C. grandiflorum* leaf extract (100 mg/ml) and amoxicillin (0.1 mg/ml) were prepared for evaluation of their combined effect on *S. aureus* and *B. subtilis.* The two agents were mixed in varying ratios ranging from 0:10 to 10: 0 of *C. grandiflorum* leaf extract and amoxicillin in accordance with the continuous variation checkerboard technique (Esimone et al., 2002; Ofokansi et al., 2008). Each of the eleven combinations of these two antimicrobial agents was serially diluted (2-fold) in 3 ml of sterile water into eight places. Two millilitres each of the dilutions of the stock mixtures was seeded into 18 ml of moltenagar. After setting, the surface of the agar was then streaked with the test microorganisms. The streaked agar plates were then incubated at 37°C for 24 h. The combined effect of the antimicrobials on the test microorganisms was determined and recorded from the fractional inhibitory concentration (FIC) index. The experiment was done in triplicate to ensure reproducibility of results. The FIC index was calculated as follows (Ofokansi et al., 2008; Esimone et al., 2002):

$$\text{FIC index} = \text{FIC}_{\text{AMX}} + \text{FIC}_{\text{CGL}} \quad (1)$$

$$FIC_{AMX} = \frac{\text{MIC of amoxicillin in combination with } C.\ grandiflorum}{\text{MIC of amoxicillin alone}} \quad (2)$$

$$FIC_{CGL} = \frac{\text{MIC of } C.\ grandiflorum \text{ leaf in combination with amoxicillin}}{\text{MIC of } C.\ grandiflorum \text{ leaf alone}} \quad (3)$$

where FIC_{AMX} is the fractional inhibitory concentration of amoxicillin and FIC_{CGL} is fractional inhibitory concentration of *C. grandiflorum*.

RESULTS

The MICs of the ethanol extract of *C. grandiflorum* leaf against *B. subtilis* and *S. aureus* was evaluated to be 50.0 ± 0.5 and 25.0 ± 0.1 mg/ml, respectively while that of amoxicillin was calculated to be 0.025 ± 0.002 and 0.05 ± 0.01 mg/ml against *S. aureus* and *B. subtilis,* respectively. The recorded MIC values were the mean of three replicate studies. Tables 1 and 2, respectively, show the results of the combined antimicrobial effect of the ethanol extract of *C. grandiflorum* leaf and amoxicillin against the test organisms. Table 1 shows the combined activity of ethanol extract of *C. grandiflorum* leaf and amoxicillin against *S. aureus*. Synergistic effects were recorded at AMX/CGL ratios of 7:3, 4:6, 3:7 and 2:8, additivity (8:2 and 6:4), indifference (1:9) and antagonism (9:1 and 5:5). In Table 2, synergism was recorded at AMX/CGL ratios of 9:1 and 7:3; antagonism (5:5 and 1:9), and indifferent effect (8:2, 6:4, 4:6, 3:7 and 2:8) against *B. subtilis*.

DISCUSSION

It could be seen from the MIC results that whereas amoxicillin showed very high activities against the

Table 1. The combined antibacterial effect of the ethanol extract of *C. grandiflorum* leaf and amoxicillin against *S. aureus.*

Drug combination ratio (AMX:CGL)	MIC of AMX (mg/ml)	MIC of CGL (mg/ml)	FIC of AMX	FIC of CGL	FIC index	Effect
10:0	0.0500	-	-	-	-	-
9:1	0.0900	0.0100	1.80	0.20	2.00	Ant
8:2	0.0400	0.0100	0.80	0.20	1.00	Add
7:3	0.0175	0.0075	0.35	0.15	0.50	Syn
6:4	0.0300	0.0200	0.60	0.40	1.00	Add
5:5	0.0500	0.0500	1.00	1.00	2.00	Ant
4:6	0.0100	0.0150	0.20	0.30	0.50	Syn
3:7	0.0075	0.0175	0.15	0.35	0.50	Syn
2:8	0.0075	0.0175	0.10	0.40	0.50	Syn
1:9	0.0050	0.0450	0.45	0.90	1.35	Ind
0:10	-	0.0500	-	-	-	-

Syn, Synergism; Ind, indifference; Add, additivity; Ant, antagonism; MIC of AMX and CGL evaluated from agar dilution method against *S. aureus* were 0.025 ± 0.002 and 25.0 ± 0.1 mg/ml, respectively.

Table 2. The combined antibacterial effect of the ethanol extract of *C. grandiflorum* leaf and amoxicillin against *B. Subtilis.*

Drug combination ratio (AMX:CGL)	MIC of AMX (mg/ml)	MIC of CGL (mg/ml)	FIC of AMX	FIC of CGL	FIC Index	Effect
10:0	0.0500	-	-	-	-	-
9:1	0.0225	0.0025	0.45	0.20	0.65	Syn
8:2	0.0400	0.0100	0.80	0.80	1.6	Ind
7:3	0.0175	0.0075	0.35	0.60	0.95	Syn
6:4	0.0150	0.0100	0.30	0.80	1.1	Ind
5:5	0.0500	0.0500	0.60	4.00	4.6	Ant
4:6	0.0100	0.0150	0.20	0.12	1.4	Ind
3:7	0.0075	0.0175	0.15	1.40	1.55	Ind
2:8	0.0050	0.0200	0.10	1.60	1.7	Ind
1:9	0.0050	0.0450	0.02	3.60	3.62	Ant
0:10	-	0.0125	-	-	-	-

Syn, Synergism; Ind, indifference; Add, additivity; Ant, antagonism; MIC of AMX and CGL evaluated from agar dilution method against B. subtilis were 0.05±0.01 and 50.0 ± 0.5 mg/ml, respectively.

Gram-positive organisms, *S. aureus, B. subtilis* (Chambers, 2004), and *C. grandiflorum* leaf extract showed only a marginal activity against the two organisms. The FIC index is interpreted as synergism if its value is less than 1.0 additivity if it is equal to 1.0, indifference if more than 1.0 but less than 2.0 and antagonism if more than 2.0 (Esimone et al., 2002; Ofokansi et al., 2008). It is clear from Tables 1 and 2 that, although the combined antimicrobial effect against *S. aureus* and *B. subtilis* did not show a regular pattern, synergism was recorded at certain AMX/CGL combinations. In summary, the combined antimicrobial effect of the interaction between *C. grandiflorum* and amoxicillin was predominantly synergistic against *S. aureus*. This could be seen to mean a potentiation of the effect of amoxicillin (a penicillinase-susceptible penicillin) against *S. aureus* (a penicillinase-producing bacterium which is also implicated in some opportunistic infections of the skin) in the presence of ethanol extract of *C. grandiflorum* leaf. A probable explanation of the enhanced activity of amoxicillin by *C. grandiflorum* leaf extract in combination is that the amoxicillin and the antimicrobial principles in ethanol extract of *C. grandiflorum* leaf may possibly have different mechanism of action or may be inhibiting two different steps in the same biosynthetic pathway of the organism resulting in an overall synergy at certain combinations. The mechanism of the synergy demonstrated with these agents is yet unknown. However, it has been noted that two antimicrobial agents may interact antagonistically if one is bacteriostatic and the other bactericidal (Nnamani et al., 2005). Amoxicillin is known to decrease the stability

of the cell wall by inhibiting both the transpeptidase and the D-alanine carboxypeptidase enzymes (Chambers, 2004) and so acted synergistically with *C. grandiflorum* leaf extract. A more critical look at Tables 1 and 2 would reveal that the combined effect of the two antimicrobial agents is not only dependent on the ratio of combination but also on the type of the test microorganism empolyed as exemplified by *S. aureus* (a penicillinase-producing Gram-positive bacterium which is also implicated in some opportunistic infections of the skin) and *B. subtilis* (a non penicillinase-producing Gram-positive bacterium). Since the predominant interaction between AMX/CGL were positive (synergism and additivity as shown in Table 1), this justifies the folkloric use as a skin sponge in treating skin infections.

Moreover, it has been established that *C. grandiflorum* leaf possessed good antimicrobial activity against *S. aureus* (Banso, 2007). The result of our investigation revealed that *C. grandiflorum* had marginal activity against *S. aureus*. The slight variation in the results of the antimicrobial studies could be related to variation in the strains of *S. aureus* employed in the studies. Furthermore, amoxicillin is inactivated by penicillinases including those produced by *S. aureus* (Chambers, 2004). Consequently, infections caused by amoxicillin resistant isolates such as *S. aureus* could be treated with some amoxicillin/*C. grandiflorum* leaf extract combinations at certain *C. grandiflorum* leaf extract concentration as occurred in the 7:3, 4:6, 3:7 and 2:8 ratios of AMX/CGL combination, bearing in mind the inefficiency of amoxicillin to Staphylococcal infections due to inactivation of the drug by some penicillinases produced by some strains of *S. aureus*.

Conclusions

The results from this investigation has provided a preliminary evidence of some kind of bacterial interaction between ethanol extract of *C. grandiflorum* leaf and amoxicillin against *S. aureus* and *B. subtilis*. There is an indication that combinations of amoxicillin and *C. grandiflorum* leaf extract may have some usefulness in chemotherapy of infections in which *S. aureus*is implicated.

Conversely, the combined effect of the interaction against *B. subtilis* may not be highly significant. In Nsukka L.G.A. of Enugu State, Nigeria, where *C. grandiflorum* leaf is commonly used as a bathing sponge in the treatment of skin infections; the possible therapeutic implications of using the leaf concomitantly with amoxicillin cannot be overlooked. Further studies would seek to evaluate the efficacy of amoxicillin/*C. Grandiflorum* leaf ethanol extract combinations as topical agents for the treatment of skin infections caused by *S. aureus* in experimental animals.

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