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Moringa oleifera leaf extract potentiates anti-pseudomonal activity of ciprofloxacin

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The aim of this study was to evaluate the *in vitro* antimicrobial interaction between the ethanol leaf extract of *Moringa oleifera* (MO), which is used in Nigeria as a dietary supplement, and ciprofloxacin (Cp), a flouroquinolone antibiotic. Preliminary antimicrobial screening of the ethanol extract of *M. oleifera* and ciprofloxacin was determined *in vitro* using the agar dilution method. The antimicrobial interaction between these agents was evaluated by the Checkerboard technique using *Staphylococcus aureus* and *Pseudomonas aeruginosa* as test organisms. The minimum inhibitory concentration (MIC) values of the extract against *S. aureus* and *P. aeruginosa* were 25.0 and 50.0 mg/mL, respectively, while that of ciprofloxacin were 0.00062 and 0.0005 mg/mL against *S. aureus* and *P. aeruginosa*, respectively. The antibacterial interaction studies indicated that the combinations predominantly showed additive effects at Cp : MO ratios of 8:2, 7:3, 6:4 and 5:5 against *S. aureus* while Cp : MO ratios of 9:1, 8:2, 7:3 and 6:4 yielded predominantly synergistic effect against *P. aeruginosa*. Other combination ratios had no MIC, hence no observed effect. This study has demonstrated that the ethanol leaf extract of *M. oleifera* possesses potent antibacterial effect against *S. aureus* and *P. aeruginosa*. Overall, the combined antimicrobial effect of the interaction between the extract and ciprofloxacin was predominantly synergistic against *P. aeruginosa*. Regarding its relevance, this study has provided a preliminary evidence of some kind of antibacterial interaction between ethanol extract of *M. oleifera* leaf and ciprofloxacin against *P. aeruginosa* and has established that the use of *M. oleifera* concurrently with ciprofloxacin would yield greater effectiveness in the treatment of infections in which *P. aeruginosa* is implicated than when either ciprofloxacin or the extract is used alone.

Key words: *Moringa oleifera* leaf, antibacterial interaction, checkerboard technique, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, ciprofloxacin.

INTRODUCTION

In tropical countries, infectious diseases account for approximately one half of all deaths and are considered major threats to human health due to unavailability of vaccines or limited chemotherapy. They continue to be a

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growing public health concern and have become the third leading cause of death since 1992, with an increase of 58% (Iwu et al., 1999; Kone et al., 2006; Pinner et al., 1996). Unfortunately, most of the current antibiotics have considerable limitations in terms of antimicrobial spectrum, side effects and their widespread overuse has led to increasing clinical resistance of previously sensitive micro-organisms and to the occurrence of uncommon infections (Cos et al., 2006; Ofokansi et al., 2013). The upsurge in side effects of many synthetic and semisynthetic anti-microbial agents in addition to multidrug resistant bacteria has spurred scientists on the research for plant-based antimicrobials of therapeutic potential (Betoni et al., 2006; Lewis and Ausubel, 2006; Lee et al., 2007).

The primary benefit of using plant-derived medicine is that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatments (Ajali and Okoye, 2009). In recent times, focus on plant research has increased all over the world and a lot of evidence has been collected to show immense potential of medicinal plants used in various traditional systems (Dahanuka et al., 2002). Plants may become the bases for the development of a new medicine or they may be used as phyto-medicine for the treatment of disease (Iwu et al., 1999). It is estimated that today, plant materials are present in, or have provided the models for 50% Western drugs (Giridhari et al., 2011). The study of antibacterial activity of medicinal plants is based on the investigation of active principles such as alkaloids, saponins, tannins, flavonoids, glycosides, vitamins and volatile oils (Iwu, 1993; Trease and Evans, 2002; Sofowora, 2008; Okore, 2009). These active principles reside in parts of plants such as the leaves, stems, barks, roots, fruits, seeds and flowers. However, certain substances (lignin, starch, cellulose and chitin) could modify or inhibit these activities of medicinal plants making it imperative to carry out extraction, characterization and identification of active principles as well as *in vitro* antimicrobial activity before proceeding to an *in vivo* trial (Okore et al., 2009; Kone et al., 2006; Cos et al., 2006).

Moringa oleifera Linn. (Family Moringaceae), also known as the horse-radish tree or drumstick tree, a rapidly-growing tree, native to Indian sub-continent, is now widely cultivated and has become naturalized in many locations in the tropics (Alam et al., 2011). The plant is rich in vitamins (A, B and C), minerals (such as calcium, potassium and iron), highly digestible proteins and carotenoids (including β-carotene or pro-vitamin A) (Fahey, 2000; Mensah et al., 2012; Dolly et al., 2009). Almost all parts of the plant have dietary as well as medicinal properties owing to its phytoconstituents. In particular, the iron content of the leaves is very good and prescribed for anaemia in the Northern Nigeria and the Philippines. The leaves are excellent sources of proteins and sulphur-containing amino-acids: methionine and cystine which are often in short supply in the plant kingdom (Mensah et al., 2012; Fozia et al., 2012). The leaf of the plant is widely used in folkloric medicine owing to its anti-tumor, hypotensive, anti-oxidant, radio-protective, anti-inflammatory and diuretic properties. *M. oleifera* has antibiotic, anti-tryponosomal, hypotensive, hypoglycemic, anti-diabetic and anti-inflammatory activities (Giridhari et al., 2011; Mensah et al., 2012). Specific phytochemicals of the plant that have been reported to possess hypotensive, anticancer and antibacterial activities include 4- (4'-O-acetyl-α-L-rhamnopyranosyloxy) benzyl isothiocy- anate, 4-(α-L-rhamnopyranosyloxy) benzyl isothiocy-anate, niazimicin, pterygospermin, benzyl isothiocyanate and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate (Fahey, 2000; Akhtar and Ahmad, 1995; Anwar and Bhanger, 2003; Asres, 1995).

Ciprofloxacin is a synthetic broad spectrum fluoro-quinolone (Ofokansi et al., 2013). It has *in vitro* and *in vivo* activities against a wide range of Gram-negative and positive aerobic and anaerobic microorganisms, including *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Chambers, 2004). Ciprofloxacin inhibits bacterial deoxyri-bonucleic acid (DNA) gyrase and topoisomerase IV, enzymes essential for bacterial replication. Inhibition of topoisomerase IV interferes with separation of the replicated chromosomal DNA into the respective daughter cells during cell division whereas inhibition of DNA gyrase prevents the relaxation of positively supercoiled DNA that is required for normal transcription and replication (Radberg et al., 1990). Concurrent use of orthodox and herbal medicines is practiced in many urban and rural communities in Africa and Asia including many communities and cities in Nigeria. It is likely that certain interactions may be taking place, without detection, in persons who have this habit of concomitant use of orthodox medicines and herbal drugs. Such interactions may result in synergistic, antagonistic, indifferent or additive effects (Ofokansi et al., 2008, 2012; Esimone et al., 2002).

A lot of work has been carried out regarding the interaction of herbal extracts and ciprofloxacin (Ofokansi et al., 2013; Esimone et al., 2002; Ofokansi et al., 2012). The interest in the present study is being spurred by our observation, over the years, that a large number of people habitually use *M. oleifera* as a dietary supplement and a good number of these people usually continue in this habit unsuspectingly even when they are placed on one kind of drug or the other including antibiotics such as ciprofloxacin. To the best of our knowledge, there has not been any reported work on the interaction between ciprofloxacin and *M. oleifera* ethanolic leaf extract. Consequently, the objective of this study was to investigate, *in vitro*, the interaction of crude ethanol leaf extract of *M. oleifera* and ciprofloxacin and their effect, in combination, on isolates of *S. aureus* and *P. aeruginosa* using the Checkerboard method. The result obtained would help to a great extent in designing a highly effective antibiotic combination against infections caused by these bacteria.

MATERIALS AND METHODS

Analytical grades of ethanol 99% (Fluka, Germany) and dimethyl-sulphoxide, DMSO (Merck, Germany) were used for extraction and dilution respectively of the *M. oleifera* leaf extract. Distilled water was collected from an all-glass still. Nutrient agar (Fluka, Germany), Mueller Hinton agar (Oxoid, England) and Nutrient broth (Biotech, Germany) were used as media for the study. Ciprofloxacin pure powder (Juhel Nig. Ltd., Nigeria) was used as synthetic antibiotic.
Cultures of *S. aureus* ATCC 1370 and *P. aeruginosa* ATCC 9648 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 *M. oleifera* were obtained in June, 2012 from Akokwa, in Isikwuato Local Governement Area of Imo State, Nigeria. Their botanical identities were determined and authenticated by Mr. A. Ozioko, a taxonomist with the International Centre for Ethnomedicine and Drug Development (INTERCEDD), Nsukka. The voucher specimen was deposited at the centre for future references.

Preparation of the *M. oleifera* leaf ethanol extract

The *M. oleifera* 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 500 g of the fine powder was extracted with 2 L of ethanol (90% v/v) using a Soxhlet apparatus. 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 and standardization of stock microbial cultures

All culture media were prepared according to the manufacturer's specification. Appropriate quantity of the media as calculated was dissolved in the required amount of solvent (distilled water). Heat was applied to aid dissolution. They were then dispensed into bijou bottles and sterilized in the autoclave at 121°C for 15 min.
The stock microbial cultures were maintained on nutrient agar slants at 4°C. For each round of experiment, the isolates were activated, by sub-culturing into 5 mL sterile nutrient broth and incubated at 37°C for 18 - 24 h. The isolates were standardized by dilution (1:100) using sterile distilled water which was a modification of the method employed by Grierson and Afolayan (1999).

Preliminary antimicrobial screening

Preliminary antimicrobial screening of the *M. oleifera* leaf extract was carried out using the agar dilution method (Ofokansi et al., 2008; Esimone et al., 2002; Ofokansi et al., 2012). Molten Mueller-Hinton agar (19 mL) in a sterile Petri dish (a plate for each dilution) was seeded with 1 mL of each of the two-fold dilution of the extract in DMSO (100, 50, 25, 12.5, 6.25 and 3.125 mg/mL) and thoroughly mixed. The agar plates were allowed to set and thereafter the plates were dried at 37°C for 1 h and a loopful of *S. aureus* broth culture was inoculated on the agar surface. The incubation was done at 37°C for 24 h and thereafter the plates were observed for growth. The experiment was repeated for *P. aeruginosa*. A control experiment was also set up against each test organism using DMSO as a control diluent. The whole experiment was similarly repeated for 100 mg/mL of ciprofloxacin using sterile distilled water as the solvent for dilution.

Determination of the minimum inhibitory concentration (MIC)

The MIC of the *M. oleifera* leaf extract was obtained using the agar dilution technique (Ofokansi et al., 2008). A stock solution of the extract (2 g/mL) was prepared by dissolving 10 g of the extract in 5 mL of 50% DMSO (one part of DMSO in one part of water). Then two-fold serial dilutions were made with sterile distilled water to obtain concentrations down to 62.5 mg/mL. A volume of each of the concentrations equal to 1 mL was transferred into an agar plate and made up to 20 mL with Mueller-Hinton 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 ciprofloxacin but in this case a stock solution of 100 mg/mL was prepared and the final concentrations obtained in agar plates ranged from 100 to 0.0001 mg/mL. Control plate having 5 mL of 50 % DMSO in 15 mL of molten agar was prepared for *M. oleifera* leaf ethanol extract. 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.

Evaluation of the interaction between *M. oleifera* leaf extract and ciprofloxacin

Two stock solutions of ciprofloxacin and *M. oleifera* leaf ethanolic extract were prepared for evaluation of their combined effect on *S. aureus* and *P. aeruginosa.* Ciprofloxacin and *M. oleifera* ethanol leaf extract solutions were prepared with DMSO in sterile test tubes, each containing twice their individual MICs (32 and 10,000 µg/mL respectively against *P. aeruginosa* and 1 and 10,000 µg/mL respectively against *S. aureus*). The two agents were mixed in varying ratios of 0:10, 1:9, 2:8……. to 10:0 of *M. oleifera* leaf extract and ciprofloxacin in accordance with the continuous variation Checkerboard technique (Esimone et al., 2002; Ofokansi et al., 2012). Each of the eleven combinations of these two antimicrobial agents was serially diluted (2-fold) in 3 mL of DMSO into eight places. A 2 mL volume of each of the dilutions of the stock mixtures was seeded into 18 mL of molten Mueller-Hinton agar. 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 FIC index was calculated as follows (Ofokansi et al., 2013):

$$FIC_{Index} = FIC_{Cp} + FIC_{ML} \quad (1)$$

$$FIC_{Cp} = \frac{\text{MIC of ciprofloxacin in combination with Moringa oleifera leaf extract}}{\text{MIC of ciprofloxacin alone}} \quad (2)$$

$$FIC_{ML} = \frac{\text{MIC of Moringa oleifera leaf extract in combination with ciprofloxacin}}{\text{MIC of Moringa oleifera leaf extract alone}} \quad (3)$$

Where Cp is the drug ciprofloxacin, M is *M. oleifera* ethanol leaf extract, FIC_{Cp} is the fractional inhibitory concentration of ciprofloxacin and FIC_{ML} is fractional inhibitory concentration of *M. oleifera* leaf extract.

RESULTS AND DISCUSSION

The MIC values of the ethanol extract of *M. oleifera* leaf

Table 1. The combined antibacterial effect of the ethanol extract of *M. oleifera* leaf and ciprofloxacin against *S. aureus*.

Drug combination ratio (Cp : MO)	MIC of Cp (µg/mL)	MIC of MO (µg/mL)	FIC of Cp	FIC of MO	FIC Index	Effect
10:0	-	-	-	-	-	-
9:1	-	-	-	-	-	-
8:2	0.0500	5000	0.80	0.20	1.00	Add
7:3	0.0438	7500	0.70	0.30	1.00	Add
6:4	0.0375	10000	0.60	0.40	1.00	Add
5:5	0.3125	12500	0.50	0.50	1.00	Add
4:6	-	-	-	-	-	-
3:7	-	-	-	-	-	-
2:8	-	-	-	-	-	-
1:9	-	-	-	-	-	-
0:10	-	-	-	-	-	-

Add = Additivity; MIC of Cp and MO evaluated from agar dilution method against *S. aureus* were 0.00062 ± 0.0001 and 25.0 ± 0.3 mg/mL, respectively.

Table 2. The combined antibacterial effect of the ethanol extract of *Moringa oleifera* leaf and Ciprofloxacin against *P. aeruginosa*.

Drug combination ratio (Cp : MO)	MIC of Cp (µg/mL)	MIC of MO (µg/mL)	FIC of Cp	FIC of MO	FIC Index	Effect
10:0	0.5000	0.0000	1.00	0.00	1.00	Add
9:1	0.2250	2500	0.45	0.05	0.50	Syn
8:2	0.2000	5000	0.40	0.10	0.50	Syn
7:3	0.1750	7500	0.35	0.15	0.50	Syn
6:4	0.1500	10000	0.30	0.20	0.50	Syn
5:5	-	-	-	-	-	-
4:6	-	-	-	-	-	-
3:7	-	-	-	-	-	-
2:8	-	-	-	-	-	-
1:9	-	-	-	-	-	-
0:10	-	-	-	-	-	-

Syn = Synergism; Add = Additivity; MIC of Cp and MO evaluated from agar dilution method against *P. aeruginosa* were 0.0005 and 50.0 mg/mL, respectively.

against *S. aureus* and *P. aeruginosa* were determined to be 25.0 and 50.0 mg/mL respectively while that of ciprofloxacin was calculated to be 0.00062 and 0.0005 mg/mL against *S. aureus* and *P. aeruginosa*, respectively. Tables 1 and 2 show the results of the combined antimicrobial effect of the ethanol extract of *M. oleifera* leaf and ciprofloxacin against the test microorganisms.

Table 1 shows the combined activity of ethanol extract of *M. oleifera* leaf and ciprofloxacin against *S. aureus*. The combinations predominantly showed additive effects at Cp : MO ratios of 8:2, 7:3, 6:4 and 5:5. In Table 2, additivity was observed in the combination ratio of 10:0 (Cp/MO) while other combinations (9:1, 8:2, 7:3 and 6:4) yielded predominantly synergistic effect against *P. aeruginosa*. Other combining ratios could show antagonism or even indifference against the test organisms but this was not observed since no MIC was obtained from such combination ratios.

Antimicrobial substances are desirable tools in the control of undesirable microorganisms especially in the treatment of infections and in preservation of food. The active components usually interfere with the growth or metabolism of microorganisms in a negative manner (Ofokansi et al., 2013).

The preliminary sensitivity screening shows that the ethanol extract of *M. oleifera* leaf possesses activity against *S. aureus* (a Gram positive bacterium) and *P. aeruginosa* (a Gram negative bacterium) (Chambers, 2004). The effect produced by the ethanol extract of *M. oleifera* leaf is however lower than that of the standard drug (ciprofloxacin). This suggests that higher concentrations of the extract could produce comparable antimicrobial results. The antimicrobial activity of an agent is usually quantified by determining the MIC values which serve as a guide for treatment of most infections (Ofokansi et al., 2012). Thus, the result of the preliminary antimicrobial screening was further supported by the MICs of the extract which were 25.0 and 50.0 mg/ml against *S. aureus* and *P. aeruginosa*, respectively. This shows that the active principles of the *M. oleifera* leaf are

active against the test organisms, consistent with previous studies (Abdulmoneim and Abu, 2011; Karthy et al., 2009; Suarez et al., 2005; Fisch, 2004). The MIC results indicate that unlike the ethanol extract of *M. oleifera* leaf, which showed only a marginal activity against *S. aureus* and *P. aeruginosa*, ciprofloxacin showed very high activities against the two organisms as expected, being a broad-spectrum highly active fluoroquinolone (Chambers, 2004; Radberg et al., 1990; Esimone et al., 2002; Ofokansi et al., 2012).

The problem of antimicrobial resistance has considerably reduced since the inception of combined antimicrobial chemotherapy; hence, the combination of two or more antimicrobials has for many years, been recognized as an important method for preventing or at least delaying bacterial resistance (Ofokansi et al., 2013, 2012). In this study, the Checkerboard method was adopted for the evaluation of the antibacterial effects of ciprofloxacin and ethanolic leaf extract of *M. oleifera* and fractional inhibitory concentration (FIC) index was used to assess the nature of the observed effects. 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 it is more than 1.0 but less than 2.0 and antagonism if it is more than 2.0 (Ofokansi et al., 2008; Esimone et al., 2002). A more critical look at Tables 1 and 2 would reveal that the combined effect of the two antimicrobial agents depends on the type of the test microorganism employed as exemplified by *P. aeruginosa* and *S. aureus*. It is clear from Tables 1 and 2 that, while the combined antimicrobial effect against *S. aureus* was predominantly additive, synergism was recorded in most of the Cp : MO combinations against *P. aeruginosa*. It was equally observed that the synergy and additivity recorded for combinations of ciprofloxacin and *M. oleifera* leaf ethanol extract against *P. aeruginosa* and *S. aureus* respectively was independent of the ratios of the combination. However, it is discernible from Table 2 that the highest potency of *M. oleifera* ethanol leaf extract in combination was found at MIC combinations of 250:0.225 (*Moringa* : ciprofloxacin), where the MIC of the extract was reduced by 200-fold. This implies that *M. oleifera* ethanol leaf extract is most active at this concentration against *P. aeruginosa*. For this plant extract, it is possible that the antibacterial principles reside within the secondary metabolites and the effects are more pronounced when used together than when used singly. A probable explanation of the enhanced activity in combination of CP : MO, particularly the potentiation of the effect of ciprofloxacin on *Pseudomonas aeruginosa* by *M. oleifera* is that the ciprofloxacin and the antimicrobial principles in ethanol extract of *M. oleifera* leaf may possibly have same mechanism of action or may be inhibiting a common step in the same biosynthetic pathway of the organism resulting in an overall synergy at certain combinations. Ciprofloxacin is known to act by preventing bacterial replication through inhibition of DNA gyrase. Although the mechanism of action of *M. oleifera* leaf extract is yet to be completely elucidated, pterygospermin, the main active constituent of *M. oleifera* has severally been reported to be responsible for its antimicrobial activity (Giridhari et al., 2011; Fahey, 2000; Mensah et al., 2012; Fozia et al., 2012; Anwar and Bhanger, 2003). More so, it has been documented that *M. oleifera* inhibits transaminase, an important enzyme in bacterial protein synthesis (Abduhnoneim and Abu, 2011; Karthy et al., 2009; Suarez et al., 2005; Fisch, 2004). Since both drugs target cellular activity, synergism or at least additivity is expected. The accumulation of both drugs in the cell could be responsible for the synergistic/additive effect observed at certain combination ratios. However, it has been noted that two antimicrobial agents may interact antagonistically if one is bacteriostatic and the other is bactericidal (Betoni et al., 2006).

Moreover, synergy was observed in most of the combination ratios with *M. oleifera* ethanol leaf extract against *P. aeruginosa* indicating that the organism is more sensitive than *S. aureus* to the leaf extract of *M. oelifera*. This could be seen to mean a potentiation of the effect of ciprofloxacin against *P. aeruginosa* in the presence of ethanol extract of *M. oleifera* leaf. The results suggest that it could be more therapeutically beneficial to use the combined extract and ciprofloxacin against infec-tions caused by *P. aeruginosa*, an opportunistic, nocosomial pathogen of immuno-compromised individuals, which not only colonizes medical devices (e.g., catheters) and infects the pulmonary tract, urinary tract, burns, wounds but also causes blood infections, infections of burn injuries and of the outer ear (otitis externa) (Ofokansi et al., 2013; Abduhnoneim and Abu, 2011; Karthy et al., 2009; Suarez et al., 2005; Fisch, 2004). In that case, greater antibacterial effect could be obtained at lower doses of each agent thereby minimizing their possible adverse effects and resistance of *P. aeruginosa* to these agents.

Conclusions

In conclusion, combination chemotherapy is clinically adopted to achieve a broad-spectrum coverage of invading organisms and to prevent the emergence of resistant organisms. Owing to the variability in the characteristics of microorganisms to antimicrobial agents, and their combinations, the clinical application of any combination requires the prior *in vitro* determination of the usefulness of the combination in any particular disease state. This study has provided a preliminary evidence of some kind of antibacterial interaction between ethanol extract of *M. oleifera* leaf and ciprofloxacin against *P.aeruginosa* and has established that the use of *M. oleifera* concurrently with ciprofloxacin would yield greater effectiveness in the treatment of infections in which *P. aeruginosa* is implicated than when either ciprofloxacin or the extract is used

alone. The combined effect of the interaction against *S. aureus* may not be highly significant at some ratios of combination of ciprofloxacin and the ethanol extract of *M. oleifera* leaf. Further *in vivo* studies would be required to assess the potential usefulness of these preliminary results in real infectious states when *P. aeruginosa* or *S. aureus* is the invading bacterium.

Conflict of Interests

The author(s) have not declared any conflict of interests

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