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Combination studies of black pepper and marketed brands of ciprofloxacin on human pathogenic organisms

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ABSTRACT

Along the coastal region of Africa especially Nigeria it is traditional practice to use pepper soup alongside prescribed antibiotics for the treatment of upper respiratory infections. This study was designed to investigate the effect of combining the bioactive spice, *Piper guineense* (black pepper) with ciprofloxacin to determine additive, synergistic or antagonistic effects. Three brands of ciprofloxacin namely Ciprotab, M & B Cipro and Ciprogem were tested for their activity against *Bacillus subtilis, Staphylococcus* species*, Escherichia coli, Enterobacter agglomerans, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Acinetobacter baumanii.* The minimum inhibitory concentrations (MIC) of the extract of the black pepper and antibiotics were determined against the indicator organisms using the agar dilution method on Mueller-Hinton agar. The findings of this study showed that Ciprogem was the most effective brand of the tested ciprofloxacin with MIC ranging from 0.25 mg/ml to 125 mg/ml against *Staph.* sp. and *Klebsiella pneumoniae*. Also, there was a synergistic effect between the extract of black pepper at 1.25 % (^{V}/v) and 0.63 % (^{V}/v) and ciprogem with MIC of ciprogem decreasing from 15.63 mg/ml to 3.90 mg/ml and 1.95 mg/ml respectively against *Escherichia coli*.

Keywords: Black pepper, Ciprofloxacin, Agar dilution, Minimum inhibitory concentration

INTRODUCTION

Herbs are safe, less toxic, economical and a reliable key natural resource of drugs all over the world (Kavitha and Lakshmi, 2016). Use of traditional medicine among the tribal local people and medicinal healers is significant and still widely practiced till date (Ekor, 2014; Oyinlola et al., 2016). The volatile oil of *Piper guineense* (black pepper) has been shown to have antimicrobial property (Omodamiro and Ekeleme 2013). Black pepper has been shown to have antipyretic properties. Its other medicinal properties include treating vertigo, arthritic disorders, diarrhea and cholera (Morufu et al., 2016). Sara et al. (2015) reported the use of black pepper for the treatment of constipation, ear ache, gangrene, heart disease, hernia, hoarseness, indigestion, insect bites, insomnia, joint pain, liver problems, lung disease, oral abscesses, sunburn, tooth decay, and toothaches.

Its broad spectrum of activity, excellent tissue penetration, and availability forms (oral and intravenous) make ciprofloxacin an excellent antibiotic derived from fluoroquinolone (Laurence et al., 2005). It is active against most strains of bacterial pathogens responsible for respiratory, urinary tract, gastrointestinal, and abdominal infections, including Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*), and Gram-positive (methicillin-sensitive but not methicillin-resistant *Staphylococcus aureus*).

Antimicrobial compounds used in combination might promote the effectiveness of each agent, with efficacy being achieved using a lower dose of each drug. Pharmacological benefits would accrue, with one drug clearing infection from one body system while the other clears it from a different site (Osonwa et al., 2017). Plant antimicrobials have been found to be synergistic enhancers in that though they may not

have any antimicrobial properties alone, but when they are taken concurrently with standard drugs they enhance the effect of those drugs (Kamatou et al., 2006). Combination therapy can be used to expand the antimicrobial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity and to obtain synergistic antimicrobial activity, it could be an alternative to monotherapy for patients with invasive infections that are difficult to treat, such as those due to multi-resistant species and for those who fail to respond to standard treatment (Kamatou et al., 2006). A number of compounds with an *in vitro* activity of reducing the MICs of antibiotics against resistant organisms have also been isolated from plants. Diterpenes, triterpenes, alkyl gallates, flavones and pyridines have been reported to have resistance modulating abilities on various antibiotics against resistant strains of *S. aureus* (Oluwatuyi et al., 2004; Smith et al., 2007).

Infectious diseases remain a major cause of death worldwide. A remarkable situation is observed in developed countries where infectious disease mortality rates are on the increase. These increases are attributed to increases in respiratory tract infections and antibiotic resistance in both nosocomial and community acquired infections (Malhotra-Kumar et al., 2016). These negative health trends call for a renewed interest in infectious diseases in the medical and public health communities and renewed strategies on treatment and prevention. This study was therefore designed to determine the antimicrobial activity of black pepper and ciprofloxacin (Ciprotab, M & B Cipro and Ciprogem) and also to investigate the combined effect of the spice extract and the tested brands of antibiotic *in vitro.*

METHODOLOGY

Three different marketed brands of the antibiotic (ciprofloxacin) of known potency availed from reputable manufacturers were examined: Ciprogem, Ciprotab and M & B Cipro. Oxoid Mueller-Hinton Agar CM0337 was used. Ethanol was distilled at the boiling point of 78 °C. Sterilin Petri dishes were used.

Plant identification, collection and processing

The plants (black pepper) was bought from an herb vendor at Ketu market, Agboyi-Ketu Local Council Development Area, Lagos State, Nigeria for authentication at the herbarium section of the Department of Botany, Faculty of Science, University of Lagos and specimen sample deposited.

Piper guineense LUH 6279 (black pepper)

The plant was commercially obtained afterwards from Agege market, Agege Local Government, Lagos, Nigeria. These were air-dried for several days until completely dried and then reduced to powder using a local grinding mill.

Plant extraction

This was done according to the method described by Gorgani et al. (2017) with modification. Two hundred grams (200 g) of powdered black pepper was loaded into the thimble inside the Soxhlet apparatus or extractor plugged with cotton wool. The apparatus was connected to a condenser above and to a round-bottom flask below containing the solvent, 2.5 liters absolute ethanol. The solvent was heated using an isomantle (water bath) at 70 – 80 ° C. Continuous extraction was done until the condensate turned colourless which signified that the powdered-spice had been exhaustively extracted. After exhaustive extraction, the solvent (absolute ethanol) was recovered using a rotary evaporator. The extract was then concentrated *in vacuo.* The test organisms were collected from culture bank of the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria. The extract yield was 9.30 g.

Determination of Minimum Inhibitory Concentration (MIC) of crude extracts against selected pathogens

The MICs were determined according to standard methods Nayef (2016). Briefly, different dilutions were prepared to give concentrations of 50, 25, 12.5 and 6.25 per cent volume by volume (% ^{V}/v). One (1) mL of each dilution of the extract was mixed with 19 mL of sterile Mueller-Hinton agar (1 in 20 dilution) to give final concentrations of 5, 2.5, 1.25 and 0.625 (% ^{V}/v) respectively, poured into Petri dishes and allowed to set. The surface of the set agar plates were allowed to dry before streaking with an overnight broth culture adjusted to 0.5 Mac Farland standards (10^8 CFU/ml) of the selected isolates and then incubated appropriately. The plates were then examined for the presence/absence of growth.

of growth. The lowest concentration of the extract inhibiting the visible growth of each organism on the agar plate was regarded as the minimum inhibitory concentration. All experiments were conducted in triplicates.

Determination of Minimum Inhibitory Concentration (MIC) of antibiotics

This was done according to standard (Nayef, 2016). Briefly, two (2) tablets were ground aseptically and dissolved in 20 mL sterile distilled water at 50 mg/mL and thus regarded as stock solution. A ten-fold dilution was made from the stock solution to yield a concentration of 5 mg/mL. From this dilution double-fold dilutions were up to 15 mg/mL. One (1) mL of each dilution was mixed with 19 mL of molten Mueller-Hinton agar (1 in 20 dilutions) to give final concentrations of 0.015, 0.03, 0.06, 0.12, 0.244, 0.488, 0.976, 1.953, 3.90, 7.813, 15.625 up to 250 µg/mL and stock solution, poured into Petri dishes and allowed to set. The surface of the set agar plates were allowed to dry before streaking with an overnight broth culture adjusted to 0.5 Mac Farland standards of the bacterial isolates and then incubated in air at 37 °C for 24 hr after which the cultures were observed for growth or not. The plates were then examined for the presence/absence of growth. The lowest concentration inhibiting the visible growth of each organism on the agar plate was regarded as the minimum inhibitory concentration.

Combination studies on selected isolates

Combination study was done according to standard procedure (Adeniyi et al., 2000). Ciprofloxacin (ciprogem) and black pepper were combined against the selected isolates: *Staphylococcus* species*, Klebsiella pneumoniae* and *Escherichia coli.*

These isolates are responsible for respiratory, urinary, gastrointestinal tracts, and abdominal infections. The sub-MIC of the antibiotic was used as the stock concentration for each bacterial isolates. 66.6 (% ^{V}/v) concentration of the extract was prepared. The stock solution for each isolate was then supplemented with 1 mL of 66.6 (% ^{V}/v) concentration of the extract.

Stepwise dilutions were made by aseptically transferring 1 mL of the previous dilution into 1 mL sterile distilled water up to the forth dilution and then 1 mL of each was introduced aseptically into 14 mL sterile Mueller-Hinton agar. Each dilution was poured into petri dishes and allowed to set.

The surface of the set agar plates were allowed to dry before streaking with an overnight broth culture equivalent to 0.5 Mac Farland standards of the isolates and then incubated appropriately. The plates were then examined for growth. All experiments were performed in triplicates.

RESULTS

The minimum inhibitory concentration of spice extract against selected isolates expressed in volume by volume per cent (^{V}/v) % is represented in Table 1 and that of the three brands of ciprofloxacin individually against tested isolates expressed in milligram per milliliter (mg/ml) are indicated in Tables 2 - 4. Table 5 shows the MIC of all three brands of antibiotics (ciprofloxacin) against tested isolates together at a glance.

Combination study was carried out on ciprogem being the most effective of all tested brands of antibiotic against the tested isolates in this present study and black pepper as it possesses some antibacterial activity. The combination study was carried out against three bacterial isolates to include *Staphylococcus* sp., *Escherichia coli* and *Klebsiella pneumoniae*. The combined study revealed a reduction in the MIC of ciprogem against *Escherichia coli* from 15.63 mg/mL to 3.90 mg/mL represented in Table 6.

DISCUSSION

There have been previous reports (Chalkley and Koornhof, 1985) of MIC value of 0.25 mg/ml against *Staphylococcus* sp. which was also observed in this study for ciprogem, ciprotab and M & B cipro. For *Klebsiella pneumoniae,* MIC was 125 mg/ml for ciprogem and M & B cipro and 250 mg/ml (*Klebsiella pneumoniae*) for ciprotab from this study.

Ciprofloxacin as evaluated here is particularly effective against Gram-negative bacteria at relatively low concentrations with minimum inhibitory concentration ranging from 0.98 mg/ml for *Enterobacter agglomerans* (ciprogem and M & B cipro) and 1.95 mg/mL (ciprotab) to 15.63 mg/mL for *Escherichia coli* and *Pseudomonas aeruginosa* for all three brands of tested antibiotic. The minimum inhibitory concentration against *Klebsiella pneumoniae* was

somewhat high: 125 mg/mL (ciprogem) and as high as 250 mg/mL (ciprotab and M & B cipro), as compared to other Gram negative bacterial isolates probably due to bacterial resistance to ciprofloxacin (Naber et al., 2001; Wagenlehner and Naber, 2005). The different formulations of the tested

Table 1: Minimum inhibitory concentration of the extract of *Piper guineense* (Black pepper) against selected isolates

	Concentration (% ^{v}/v)							
Organism	5	2.5	1.25	0.625	0.32	0.16	0.08	MIC
E. coli	-	-	+	+	+	+	+	2.5
K. pneu.	-	+	+	+	+	+	+	5
Staph. sp.	-	-	+	+	+	+	+	2.5

+ = growth, - = no growth**,** *E. coli = Escherichia coli*, *K. pneu. = Klebsiella pneumonae***,** *Staph*. sp. = *Staphylococcus* sp.

Table 2: Minimum inhibitory concentration (MIC) of ciprogem against bacterial isolates

	Concentration (mg/ml)														
Organism	1000 (stock)	62.5	31.25	15.63	7.82	3.90	1.95	0.98	0.49	0.25	0.12	0.06	0.03	0.015	MIC
B. sub.	-	-	-	-	-	-	-	-	+	+	+	+	+	+	0.98
Staph. sp.	-	-	-	-	-	-	-	-	-	-	+	+	+	+	0.25
Kleb. pneu.	-	+	+	+	+	+	+	+	+	+	+	+	+	+	125
A. baumanii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND
Entero. agg.	-	-	-	-	-	-	-	-	+	+	+	+	+	+	0.98
Ps. aeru.	-	-	-	-	+	+	+	+	+	+	+	+	+	+	15.63
E. coli	-	-	-	-	+	+	+	+	+	+	+	+	+	+	15.63

+ = growth; - = no growth; *E. coli = Escherichia coli*; *B. sub. = Bacillus subtilis; Staph* sp. = *Staphylococcus* species*; Kleb. pneu. = Klebsiella pneumoniae*; *A. baumanii = Acinetobacter baumanii; Entero. agg. = Enterobacter agglomerans*; *Ps. aeru. = Pseudomonas aeruginosa*
ND = Not determined

Table 3: Minimum inhibitory concentration of ciprotab against bacterial isolates

	Concentration (mg/ml)														
Organism	1000 (stock)	62.5	31.25	15.63	7.82	3.90	1.95	0.98	0.49	0.25	0.12	0.06	0.03	0.015	MIC
B. sub.	-	-	-	-	-	-	-	+	+	+	+	+	+	+	1.95
Staph. sp.	-	-	-	-	-	-	-	-	-	-	+	+	+	+	0.25
Kleb. pneu.	-	+	+	+	+	+	+	+	+	+	+	+	+	+	250
A. baumanii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND
Entero. agg.	-	-	-	-	-	-	-	+	+	+	+	+	+	+	1.95
Ps. aeru.	-	-	-	-	+	+	+	+	+	+	+	+	+	+	15.63
E. coli	-	-	-	-	+	+	+	+	+	+	+	+	+	+	15.63

+ = growth; - = no growth; *E. coli = Escherichia coli*; *B. sub. = Bacillus subtilis; Staph* sp. = *Staphylococcus* species*; Kleb pneu. = Klebsiella pneumoniae*; *A. baumanii = Acinetobacter baumanii; Entero. agg. = Enterobacter agglomerans*; *Ps. aeru. = Pseudomonas aeruginosa*
ND = Not determined

It is inferred that black pepper plant crude extract contains such compounds as tannins, alkaloids, flavonoids, saponin, terpenes and sterols) that can enhance the activity of the antibiotic (Aizpurua-Oliazola et al., 2015). Growth was observed at all concentrations tested for *Staphylococcus* sp. and *Klebsiella pneumoniae* but only at 0.98 mg/ml and 1.95 mg/ml for *Escherichia coli*. Many researchers (Lawal et al., 2011; Jaruporn et al., 2017) have also reported a number of compounds (to include terpenes and flavones) with an *in vitro* activity of reducing the MICs of antibiotics against resistant organisms. Terpenes and flavones present in black pepper may thus be referred to as adjuvants.

The ability of crude extracts of plants to potentiate the activity of antibiotics has been observed by some researchers (Aiyegoro et al., 2008b, 2009). Combinations of antimicrobials that demonstrate an *in vitro* synergism against infecting strains are more likely to result in successful therapeutic result. It has been proven that, in addition to the production of intrinsic antimicrobial compounds, plants also produce Multi-Drug Resistance (MDR) inhibitors which enhance the activity of the antimicrobial compounds (Stermitz et al., 2000a).

Plant antimicrobials have been found to be synergistic enhancers in that though they may not have any antimicrobial properties alone, but when they are taken concurrently with standard drugs they enhance the effect of that drug (Kamatou *et al*., 2006). Betoni *et al*. (2006) observed synergistic interactions between extracts of Brazilian medicinal plants and eight antibiotics on *S. aureus.* Boik (2001) conducted a large number of combination studies using various natural substances and their results strongly suggested that when used in combination, natural substances can produce synergistic effects.

It is thought that phenolic compounds such as flavonoids may increase the biological activity of other compounds by synergistic or other mechanisms (Williamson, 2001). Experimental evidence of synergistic actions between plants was also shown in a clinical study on the formulation of Chinese herbs used to treat eczema (Williamson, 2001)

Table 4: Minimum inhibitory concentration of M & B cipro against bacterial isolates

Organism	Concentration (mg/ml)														
	1000 (stock)	62.5	31.25	15.63	7.82	3.90	1.95	0.98	0.49	0.25	0.12	0.06	0.03	0.015	MIC
B. sub.	-	-	-	-	-	-	-	-	+	+	+	+	+	+	0.98
Staph. sp.	-	-	-	-	-	-	-	-	-	-	+	+	+	+	0.25
Kleb. pneu.	-	+	+	+	+	+	+	+	+	+	+	+	+	+	250
A. baumanii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND
Entero. agg.	-	-	-	-	-	-	-	-	+	+	+	+	+	+	0.98
Ps. aeru.	-	-	-	-	+	+	+	+	+	+	+	+	+	+	15.63
E. coli	-	-	-	-	+	+	+	+	+	+	+	+	+	+	15.63

+ = growth; - = no growth; *E. coli = Escherichia coli*; *B. sub. = Bacillus subtilis; Staph* sp. = *Staphylococcus* species*; Kleb pneu. = Klebsiella pneumoniae*; *A. baumanii = Acinetobacter baumanii; Entero. agg. = Enterobacter agglomerans*; *Ps. aeru. = Pseudomonas aeruginosa,* ND = Not determined

Table 5: Minimum inhibitory concentration (MIC) of the three brands of ciprofloxacin (500 mg) against bacterial isolates

Organism	MIC (mg/mL)		
	Ciprogem	Ciprotab	M & B Cipro
Escherichia coli	15.63	15.63	15.63
B. sub.	0.98	1.95	0.98
Staph. sp.	0.25	0.25	0.25
Kleb. pneu.	125	250	250
A. baumanii	ND	ND	ND
Entero. agg.	0.98	1.95	0.98
Ps. aeru.	15.63	15.63	15.63

ND = No ND = Not determined

Table 6: Antimicrobial susceptibility pattern of *Escherichia coli* to the combination of antibiotic (ciprogem) and black pepper

Conc. (mg/ml) Ciprogem	Conc. (% ^{V}/v) Black Pepper	Concentration (mg/ml)
15.63	1.25	3.90
3.90	0.63	1.95
1.95	0.32	0.98
0.98	0.16	0.48

CONCLUSION

It was deduced from this study that *Klebsiella pneumoniae* was resistant to ciprofloxacin when tested alone and in combination with black pepper, while a synergistic effect of the extract of black pepper and ciprofloxacin was observed against the *Escherichia coli*. Synergy between bioactive plant product and antibiotics will confront problems of toxicity and overdose since lesser concentrations of two agents in combination are required, due to these reasons, there is need therefore, for continuous exploration of multidrug resistance modulating principles from plants sources.

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