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Evaluation of the *in-vitro* Combination Effects of Some Common Antibiotics on the Antibacterial Activity of *Hippocratea africana* Root Extracts in Folkloric Herbal therapy of Infections and Diseases

[*1]Ekong, U. S., [1]Ubulum, P. M. E., [2]Udoh, D. I., and [1]Nnatu, C. M

[1]Pharmaceutical Microbiology/Biotechnology Unit, Department of Pharmaceutics/ Pharmaceutical Technology, Faculty of Pharmacy, University of Uyo, Nigeria.

[2]Department of Microbiology, Faculty of Science, University of Uyo, Nigeria.

ABSTRACT

Antibacterial activities of *Hippocratea africana* root extracts and their combinations with some standard antibiotics were evaluated by activity index profile (AIP) technique to ascertain the efficacy of such practices in folkloric-herbal therapy of infectious- diseases. Antibacterial activities of aqueous , methanol and ethanol extracts of *H. Africana,* assessed by agar-well diffusions technique, indicated broad-spectrum of activity significantly ($P<0.05$) highest for the aqueous than the other extracts. Minimum inhibitory concentration (MIC), of the extracts determined by the reference standard agar-dilution technique (ADT, indicated predominantly low MIC values: aqueous (1.57–12.5mg/ml); ethanol (6.25 – 25.0mg/ml and methanol (12.5 –25.0 mg/ml) and correspondingly low MBC values, indicating time- dependent bactericidal activities. combination of the extracts with the common standard antibiotics at sub-inhibitory concentrations (I x MIC), assessed by the agar-well diffusion technique, indicated remarkable antibacterial comparable to the single agents. The outcome/nature of the combination empirically determined by the AIP, predominantly showed synergism, few additivity or indifference and no antagonism.

Keywords: Combination Effects, Antibiotics, Antibacterial Activity, *Hippocratea africana*, Folkloric Herbal therapy.

INTRODUCTION

Many antimicrobics whether of microbial, animals or plants origin, ever since discovered either singly or in combinations have had wider application as chemotherapeutic agents in the treatment, prevention and control of myriads of infections and diseases. Thus, combined antimicrobial therapy had long been in use. Such applications often took the advantage of different mechanisms of action and /or improved toxicity profiles of the agents (Rybak and McGrath 1996; Lampiris and Maddix, 2004; Ekong, *et .al* ., 2016). The combinations of two antimcrobics have been widely reported to often result in a significantly greater biocidal activity than the single agents (Lorian, 1991; Akunyili and Akubue, 1995; Ekong *et al*., 2008; 2010; 2015; Ekong and Ibezim, 2011, Ekong *et al*., 2016). The rationale for such combinations as previously reported include: effectiveness in the treatment of infections due to multiple organisms; initial therapy of serious infections or unknown etiology; checking the development of resistance; as well as to lessen dose-related toxicity in the case of monotherapy (Ekong and Ibezim,2015; Ekong *et al*, 2015, 2016). Generally, the therapeutic outcomes of such combinations could be synergistic, additivity, indifferent or antagonistic (Sande, 1990; Lorian, 1991; Tatro, 1992; Akunyili andAkunbue; 1995; Harter 1995; Sabbath and Lorian 1997; Ekong and Ibezim, 2015; Ekong, *et al.*, 2015; 2016).

The use of medicinal plants in the traditional medical practices had been widely reported to be potent against microorganisms, invariably due to the abundant presence of bioactive phytoconstituents, thereby constituting a rich pool of relatively untapped natural remedies and agents for the prevention and treatment of numerous ailments, infections and diseases (Harborne 1981; Iwu, 1984; Iwu*et al.*, 1999;Trease and Evans,2002; Farombin, 2003; Fabricant andFarnworth, 2001; NNMDA, 2006; Sofowora, 2008). One of the such highly potent plant with excellent antimicrobial activity and low toxicity is *Hippocratea africana* (wild), Loess (Hippocrateaeae) (Okokon*et al* 2006; 2011; Ekong and Nnatu, 2016; Ekong and Effiong, 2016; Ekong and Ubulom, 2016). *H.africana* is used by the locals in the treatment of various ailments, infections and diseases including fever, malaria, body pains, diarrhoea and other gastroenteritis, where few validations of these folkloric claims have been reported (Okokon *et al.*, 2006; 2011; Ekong and Nnatu, 2016; Ekong and Effiong, 2016; Ekong and Ubulom, 2016). However, in folkloric medical practices, due to the intractable problems of antimicrobial resistance by some hitherto susceptible microorganisms, especially the "enterics"; *H. Africana* extracts are often administered in combination with antibiotics, especially chloramphenicol to boost therapy. Alas, the scientific outcomes or implications of these folkloric adventures are not validated to ascertain the efficacy in empirical chemotherapy of infections and infectious-diseases, particularly those of gastroenteritis. Thus, there is an inevitable dearth of information on the scientific and empirical outcomes of the combination of *H. africana*, especially the root extracts with standard antibiotics in the course of treatment, and prevention and control of infections and diseases.

***Corresponding Author: ubsa4@yahoo.com.**

To this end, this research work was carried-out to validate and assess the effect of the interactions of standard antibiotics on the antibacterial activity of *H. africana* root extracts. The *H. africana* root extracts were selectively chosen for the scientific validation of such interactions, given the previously reported comparative higher photochemistry, antimicrobial activity/potency as well as least toxicity profile with respect to the *H.africana* leaf extracts (Ekong and Nnatu 2016; Ekong and Effiong, 2016; Ekong and Ubulom, 2016).

MATERIAL AND METHODS

Plants Materials, Extraction and Phytochemical Test

Fresh roots of *H. africana* plants were air-dried and pulverized with pestle and mortar, weighed and stored in air-tight polyethene bags prior to extraction. Cold maceration of the pulverized roots was carried out according to the methods of Okokon *et al., (*2006) with modifications (Ekong and Nnatu, 2016). Phytochemical tests on the respective root extracts were done using standard procedures as previously reported (Harbone, 1981; Pearson, 2005; Ekong and Nnatu, 2016).

Test Organisms and Standardization of Inocula

Test organisms used in the study were stock cultures and clinical bacterial isolates, obtained from the Pharmaceutical Microbiology/Biotechnology Unit Laboratory, Department of Pharmaceutics/Pharmaceutical Technology, Faculty of Pharmacy, University of Uyo, Nigeria. They were staphylococci (*Staphylococcus aureus,* (SA); *Staphylococcus epidermidis*, (SE); *Staphylococcus saprophyticus* (SS); streptococci *Streptococcus pneumoneae*, (SP); *Streptococcus faecalis*, (SF); *Steptococcus mutans*, (SM); *Bacillus subtlis*, (BS); *E. coli (*EC);. *Pseudomonas aeruginosa, (*PA); *Salmonella typhimurium, (*ST); *Shigella dysentariae*, (SD) and *Vibrio cholera*, (VC). Inocula of these cultures were standardized by ten-fold serial dilutions to the turbidity of 0.5 McFarlardnephelometer, with an approximated cell density of 1 x 10^8cfu/ml, following the modifications of the method of Tilton and Howard, (1987); Baron and Finegold, (1990), by Ekong *et al.*, (2004) as previously reported (Ekong and Nnatu, 2016). The purity of the standardized cultures was checked by spread-plating aliquots on nutrient-agar, (NA) (Oxoid, England) and incubated under standard conditions (Ekong *et al.*, 2004; Ekong and Nnatu, 2016).

Antibacterial Properties of *H. africana* Root Extracts

Antibacterial activity spectra of the aqueous, ethanol and methanol extracts of*H. africana* root were assessed by the modified agar-well diffusion method (Ekong *et al.*,2004), on plates of Diagnostic Sensitivity Test Agar, (DSTA) (Oxoid, England),with chloramphenicol (CHL) and ciprofloxacin (CPF) as controls, as previously reported (Ekong and Nnatu,2016).Minimum inhibitory concentrations (MIC) were determined by the macrobro-thdilution technique (Tilton and Howard,1987; Baron and Finegold,1990), following the reference standard agar-dilution technique, (ADT) (Collins and Lyne, 1979), on DSTA streak-seeded plates, as previously reported (Ekong and Nnatu, 2016). Minimum biocidal concentrations (MBC) and mode of activity of the root extracts were determined by plating inocula from the non-growth MIC plates on the extracts –free DSTA and re-incubated under standard conditions. The presence of colonies indicated biostatic activity; while the absence of colonies indicated biocidal activity (Ekong and Nnatu,2016).

Combined Antibacterial Activity of *H. africana Root Extract and Antibiotics*

Evaluation of the combined antibacterial activity between *H. africana* root extracts and standard antibiotics (controls) was carried-out at 1 x MIC, by the modified agar-well diffusion technique (Ekong*et al.*, 2015). In the assay, solutions of the antibacterial agents at 1 x MIC, were mixed in equimolar ratio ($1{:}1\,^{v}/_{v}$) ,and aliquots of the mixtures were aseptically introduced into holes, aseptically bored on previously seeded DSTA plates with 0.1ml inocula of 24h old standardized cultures. The assay plates were held at 4^{o}C for 1 h and thereafter incubated at 37^{o}C for 24 h. The plates were observedfor the presence or absence of inhibition zone diameter (IZD).

Nature of Interactions of the Combined Antibacterial Activity

The nature of the interactions of the *H. africana* root extracts and the antibiotics against the test organisms was determined by the activity-profile index, (AIP) (Ekong et al., 2015), a modification of the fractional inhibitory concentration (FIC), by the checkerboard technique (Ekong *et al.*, 2008). The AIP compares the IZD of the combined activity of the agents with the activity of the more potent uncombined agent against the test cultures at sub-inhibitory concentrations. The differences obtained were expressed as the percentage change in the IZD of the standard antibiotics in the combinations, with respect to the IZD of the combined agents (Ekong *et al.*, 2015). Thereafter, the AIP values obtained were compared to and interpreted from the strip agar diffusion interaction ranges (≥ 20.0%, synergism; < 20.0%, additivity; 0.0%, indifferent; -0.0%, antagonism) specified by Okore (2009), to evaluate the nature or outcome of the interactions (Ekong *et al.*, 2016).

RESULTS AND DISCUSSION

The phytochemical composition of *H. africana* root extracts had been previously reported by the corresponding author and his co-workers (Ekong and Nnatu, 2016; Ekong and Effiong, 2016; Ekong and Ubulom, 2016). The various reports indicated the presence of alkaloids, saponins, flavonoids, cardiac-glycosides, terpenes, steroids and carbohydrates.

Accordingly, the presence of the aforementioned phytochemicals in the root extracts of the plant could have informed the broad-spectrum antimicrobial activity of the plant. The antibacterial activities of *H. africana* root extract (Table 1), indicated excellent and laudable antimicrobial properties. These include broad-spectrum of antimicrobial activity, significantly ($p<0.05$) highest in the aqueous than the other extracts, with correspondingly excellent and significant ($p<0.05$) potencies compared to the controls. Similarly, the aqueous root extract recorded predominantly lower MIC values within the range (3.13 – 6.25 µg/ml), which were biocidal in their mode of activities against the test organism, confirming the potencies of all the root extracts. The comparative antibacterial activity of *H. africana* root extracts and standard antibiotics as well as their combined antibacterial activities (Table 2), indicated remarkable inhibitory activities comparable with those of the standard antibiotics, against the Gram positive and Gram-negative bacteria tested. These excellent activities resulted in the predominantly potentiated activity of the various combinations of the different extracts with the standard antibiotics against the test organisms. Equally, the combined activities of the standard antibiotics were not so remarkably different from those of the single agents, particularly ciprofloxacin. Generally, this result corroborated the merits of antimicrobial agents combinations, as previously stated in this work (Ekong *et al.*, 2015). Thus, the frequent practice of adding these antibiotics to the extracts of *H. africana* in folkloric medical provision, given the laudable results of the study and the stipulated rationales for such combinations, is evidently justified. This is so because all the combinations recorded remarkable activities compared to the single agents against the test organisms, wit*h H. africana* which has been reported to be used for the treatment of many infections and diseases caused by multiple organisms e.g. diarrhea (Ekong and Ubulom, 2016). Secondly, in folkloric medicine, the practitioners are ignorant of the etiologic agents of infections and diseases. Hence the combination of these agents could have been an attempt to broaden and enhanced the activity of the extracts against both known and unknown pathogens, which may be resistant organisms. This adventure cum ingenuity is fairly successful in most cases.

Table 1: Antibacterial activities of *H. africana* root extracts and standard antibiotics

Test organism	Antibacterial Activity/IZD(mm)					MIC (mg/ml)					MBC (mg/ml)					MIC/MBC Index		
	***H. africana* extract**			**Controls**		***H. africana* extracts control**					***H. africana* extracts controls**					***H. africana* extract**		
	AQ	MET	ETH	CHL	CPF	AEQ	MET	ETH	CHL	CPF	AQ	MET	ETH	CHL	CPF	AQ	MET	ETH
SA	44.0	24.0	40.0	30.0	40.0	3.13	12.5	6.25	0.625	0.0625	12.5	25.0	12.5	0.25	0.125	-	-	-
SE	34.0	30.0	32.0	30.0	40.0	6.25	12.5	6.25	0.625	0.0625	12.5	25.0	25.0	0.25	0.125	-	-	-
SS	32.0	28.0	31.0	30.0	40.0	6.25	12.5	6.25	0.625	0.0625	12.5	25.0	25.0	0.25	0.125	-	-	-
SP	38.0	33.0	36.0	36.0	40.0	6.25	12.5	12.5	0.125	0.0625	25.0	50.0	25.0	0.125	0.125	-	-	-
SF	37.0	31.0	36.0	31.0	41.0	6.25	12.5	12.5	0.125	0.0625	25.0	50.0	25.0	0.125	0.125	-	-	-
SM	39.0	34.0	37.0	34.0	42.0	6.25	12.5	6.25	0.125	0.0625	25.0	50.0	25.0	0.125	0.125	-	-	-
BS	28.0	23.0	25.3	31.0	40.0	6.25	12.5	6.25	0.125	0.0625	25.0	25.0	25.0	0.25	0.125	-	-	-
EC	27.0	22.0	25.0	31.0	40.0	6.25	12.5	6.25	0.125	0.0625	12.0	25.0	12.5	0.25	0.063	-	-	-
PA	32.0	23.0	25.0	20.0	40.0	12.5	12.5	25.0	0.125	0.0625	25.0	50.0	25.0	0.25	0.063	-	-	-
ST	25.0	23.0	25.0	30.0	40.0	6.25	12.5	6.25	0.125	0.0625	25.0	25.0	25.0	0.25	0.125	-	-	-
SD	28.0	21.0	25.0	32.0	45.0	6.25	12.5	6.25	0.0625	0.0625	12.5	25.0	12.5	0.125	0.125	-	-	-
VC	28.0	22.0	25.0	30.0	45.0	6.25	12.5	12.5	0125	0.0625	12.5	25.0	12.5	0.125	0.125	-	-	-

AQ- Aqueous extracts, MET- Methanol extract, ETH- ethanol extracts,CHL= Chlorampenicol, CPF- Ciprofloxacin; (-) absence of growth (cidal activity).

Table 2: Comparative antibacterial activity at 1 x MIC of agents and their combinations

Test Organisms	Antibacterial activity of 1 x MIC(mm)					Combined antibacterial activity of 1 x MIC (mm)						
	H. africana extracts			Controls		*H. africana extracts + controls*						
	AQ	MET	ETH	CHL	CPF	AHA + CHL	AHA + CPF	MHA + CHL	MHA + CHL	EHA + CHL	EHA + CPF	CHL + CPF
SA	4.0	3.0	4.0	6.0	10.0	18.0	16.0	10.0	12.0	10.0	15.0	10.0
SE	4.0	3.0	4.0	5.0	10.0	16.0	15.0	10.0	11.0	10.0	12.0	10.0
SS	4.0	2.0	3.0	5.0	10.0	15.0	14.0	10.0	11.0	11.0	13.0	10.0
SP	8.0	5.0	6.0	8.0	10.0	10.0	10.0	8.0	10.0	10.0	10.0	12.0
SF	8.0	4.0	6.0	8.0	10.0	10.0	10.0	8.0	10.0	10.0	10.0	12.0
SM	8.0	5.0	7.0	8.0	10.0	11.0	12.0	9.0	10.0	10.0	11.0	12.0
BS	8.0	3.0	4.0	6.0	10.0	11.0	13.0	10.0	11.0	10.0	12.0	10.0
EC	6.0	3.0	4.0	7.0	10.0	14.0	16.0	9.0	11.0	8.0	10.0	10.0
PA	5.0	3.0	5.0	5.0	10.0	12.0	15.0	9.0	10.0	10.0	13.0	12.0
ST	8.0	5.0	6.0	8.0	10.0	14.0	14.0	10.0	11.0	12.0	12.0	12.0
SD	8.0	5.0	7.0	6.0	12.0	15.0	16.0	10.0	13.0	12.0	14.0	14.0
VC	8.0	6.0	8.0	5.0	12.0	15.0	16.0	10.0	13.0	12.0	15.0	1.0

AHA = Aqueous *H.africana*extract; MHA = Methanol *H. africana extracts*; EHA=ethanol *H. africana extracts*; +=combination /interaction.

Table 3: Outcome/Nature of combine antibacterial activity of 1 x MIC of agents

Test organisms	Activity index profile of combination (%)							Inference/Nature of combinations						
	AHA + CHL	AHA + CPP	MHA + CHL	MHA + CPF	EHA + CHL	EHA + CPP	CHL + CPF	AHA + CHL	AHA + CPP	MHA + CHL	MHA + CPF	EHA + CHL	EHA + CPP	CHL + CPF
SA	66.7	37.5	40.0	16.7	40.0	333	0.0	SYN	SYN	SYN	SYN	SYN	SYN	IND
SE	68.8	33.3	50.0	9.1	50.0	16.7	0.0	SYN	SYN	SYN	ADT	SYN	ADT	IND
SS	66.7	28.6	50.0	9.1	54.5	23.1	0.0	SYN	SYN	SYN	ADT	SYN	SYN	IND
SP	20.0	0.0	0.0	0.0	0.0	0.0	16.7	SYN	SYN	IND	IND	IND	IND	ADT
SF	20.0	0.0	0.0	0.0	20.0	0.0	16.7	SYN	IND	IND	IND	SYN	IND	ADT
SM	27.3	16.7	11.1	0.0	20.0	9.1	16.7	SYN	IND	ADT	IND	SYN	ADT	ADT
BS	45.5	23.1	40.0	9.1	40.0	16.7	0.0	SYN	ADT	SYN	ADT	SYN	ADT	IND
EC	50.0	37.5	22.2	9.1	12.5	0.0	0.0	SYN	SYN	SYN	ADT	ADT	IND	IND
PA	58.3	33.3	44.4	0.0	50.0	23.1	16.7	SYN	SYN	SYN	IND	SYN	SYN	ADT
ST	42.9	28.6	20.0	9.1	33.3	16.7	16.7	SYN	SYN	SYN	ADT	SYN	ADT	ADT
SD	60.0	25.0	40.0	7.7	50.0	14.3	14.3	SYN	SYN	SYN	ADT	SYN	ADT	ADT
VC	66.7	25.0	50.0	77	50.0	7.7	7.7	SYN	SYN	SYN	ADT	SYN	SYN	ADT

AIP: ≥ 20.0% (Synergism, SYN); < 20.0% (Additivity, ADT); 0.0% (Indifferent, IND); - 0.0% (Antagonism, ANT)

The nature or outcomes of the antibacterial activity of the combination of *H. africana* root extracts and the standard antibiotics (Table 3), predominantly indicated a synergistic activity for the aqueous extract and the standard antibiotics; methanol and ethanol extracts with chloramphenicol. However, methanol and ethanol extracts with ciprofloxacin as well as the antibiotics combinations resulted in less synergism, but predominantly additivity or indifferent, without any antagonism. In this study, the outcome or effect of the interactions of *H. africana* root extracts and standard antibiotics against the test organisms, assessed by the AIP are in line with those previously reported for other antibiotics - antibiotics; antibiotics-extracts of microbial, animals or plants combinations, against other test organisms by several techniques (Ekong *et al.,* 2008; Okore 2009; Ekong *et al.,* 2010, Ekong and Ibezim, 2015; Ekong *et al.,* 2015; Ekong and Ubulum, 2016). Furthermore, besides the novel concept of AIP, the nature of antimicrobics combinations had been previously reported to be informed from the mode of activity of the combining agents' (Rahal, 1975 Sande *et al.*,1990. Accordingly, interactions between bactericidal agents often results in synergism; additivity or potentiation, in that the activity of one bactericidal agent is enhanced by the other bactericidal agent. While interactions between a bacteriostatic and bactericidal or two bacteriostatic agents, often result in antagonism, as the action of the bactericidal agent is inhibited by the bacteriostatic agents, as well as two bacteriostatic agents. However, this method of evaluating antimicrobics combinations based on the mode of activity of the agents and measured by either synergism or antagonism, gave contrarily results in a study on antibiotics combinations using bactericidal and bacteriostatic agents (Ekong, and Ibezim 2015; Ekong*et al* 2015. Ekong and Ubulum, 2016). In the present study, the mode of activity of *H. africana* extract are determined to be biocidal

(Table 1), the combinations of *H. africana* root and ciprofloxacin (a standard bactericidal agent), with chloramphenicol (a standard bacteriostatic agent) have not resulted in any antagonism, but all synergism which also included addivity or potentiation. Thus, the result of this study are in line with and supported the data previously reported in the use of AIP to determine synergism and antagonsism, as well as other interactions in antimicrobics combinations (Ekong and Ibezim, 2015; Ekong*et al.,* 2015, Ekong and Ubulum, 2016). Hence, the results of this study and those previously reported are at variance with the above concept of determining synergism and antagonism based on mode of activity of agents in combinations. From the foregoing, this could be misleading and bring to the fore, the significance(s) of the simple technique of AIP, as a concept in determining synergism and antagonism in antimicrobics combinations. However, by standard definition following mechanism of actions of antimicrobics; synergism has been reported to occur by enhancement of action of one agent by another. This could possibly be through the blockade of sequential steps in a metabolic pathway; inhibition of enzymatic inactivation of one agent by another, as well as the enhanced microbial uptake of one agent by another (Lorian, 1991; Tatro, 1992). Thus, in this study following these assertions, the synergistic interactions reported could be attributed to anyone of these mechanisms of synergism.

CONCLUSION

In this paper, we have evaluated the outcome of the combination of *H. africana* root extracts with standard antibiotics as practiced in folkloric medicine. The interactions of *H. africana* root extracts with the standard antibiotics were predominantly synergistic without any recorded antagonism. The synergistic activity of the combinations is laudable and could likely be an indication to the possibility of adoption in the empirical cum herbal therapy of specific or mixed infections with *H. africana* root extracts. As recommendation, further animal studies on the pharmacokinetics and pharmacodynamics as well as the toxicological profiles of the various combinations would be conducted to ascertain the efficacy or otherwise of the relevance of this folkoric practices in clinical chemotherapy of infections and diseases.

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