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Evaluation of the combined antimicrobial activity of the aqueous and methanolic leaf extracts of *Mitracarpusvillosus* with amoxicillin

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ABSTRACT
The antimicrobial properties of the methanolic leaf extracts of *Mitracarpusvillosus* was evaluated using laboratory strains of *Klebsiella pneumonia, Salmonella paratyphi, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Candida albicans*and*Aspergillusniger*. The minimum inhibitory concentration (MIC) of the aqueous (AE) and methanolic (ME) extracts of the plant was determined against sensitive organisms using the agar diffusion method. The combined antibacterial effect of the extracts and amoxicillin was evaluated against *S. aureus* and *B. subtilis* using the checkerboard technique. Results show that the MICs of the AE and ME against *S. aureus* are 4.22 and 4.79 mg/ml respectively, while the MIC values against *B. subtilis* are 5.01 and 7.59 mg/ml respectively for AE and ME. Combinations of the extracts with amoxicillin produced mainly a synergistic effect based on the fractional inhibitory concentration (FIC) index against the test organisms.

KEYWORDS: Amoxicillin;antimicrobial properties; leaf extracts;minimum inhibitory concentration; *Mitracarpusvillosus*.

INTRODUCTION

Over the centuries, plants have served as a source of drug, food and a host of other applications to man. Since the beginning of time, man has been fascinated by nature. Plants which for centuries have been in solitude and obscurity in the forest are becoming popular as their extracts now have proven value in providing succour for the distressed. Many modern drugs are made from nature (plants) such as digitalis, artemisinin and quinine, amongst others.

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 [1-4]. 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 [4-6].

Mitarcarpusvillosus (Rubiaceae) is a common weed in upland areas from the forest to the savanna zones of tropical Asia and Africa [7]. Its common name is tropical girdlepod. The leaves of the plant are popularly used in Eastern Nigeria for the treatment of eczema. The Yoruba and Hausa in Nigeria use the herb to treat parasitic infections. In Senegal, the plant is used for the treatment of sore throat. Among the compounds isolated from the plant is psoralen which is used for the management of vitiligo and psoriasis [8]. It is widely employed in traditional medicine in West Africa for headache, toothache, amenorrhea, dyspepsia, hepatic diseases, veneral diseases and leprosy as decoction of the leaves [9]. Irobi and Daramola [10-11] had previously demonstrated that the ethanolic extract of *M. villosus* possessed good antibacterial and antifungal activity. In addition, other medicinal plants have been investigated for their antimicrobial

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properties [12-14]. Therefore, the purpose of this work was to evaluate the antimicrobial properties of the aqueous and methanolic extracts of *Mitracarpusvillosus.* The *in vitro* antibacterial effect of the leaf extracts of *M. villosus* in combination with amoxicillin is also presented.

MATERIALS

Nutrient agar, Sabouraud dextrose agar and nutrient broth (Biotech, England) were used. Distilled water was obtained from the Department of Pharmaceutics, University of Nigeria, Nsukka. All other reagents were sourced locally and were of analytical grade.

Test organisms

Laboratory strains of *Klebsiella pneumonia, Pseudomonas aeruginosa, Escherichia coli, Salmonella paratyphi, Bacillus subtilis, Staphylococcus aureus, Candida albicans*and*Aspergillusniger*were obtained from the Pharmaceutical Microbiology Laboratory, University of Nigeria, Nsukka.

METHODS

Collection of *M. villosus* leaves

Fresh leaves of *M. villosus* were collected from Nsukka in Enugu State between April and June 2005 and authenticated by Mr. A. Ozioko, a plant taxonomist of the Department of Botany, University of Nigeria. Voucher specimens were deposited in the herbarium of the University of Nigeria.

Extraction of *M. villosus* leaves

The leaves of *M. villosus* were shade-dried for five days and ground into fine powder. This was divided into two portions for extraction with methanol using Soxhlet apparatus and with water using cold maceration method. The extracts in each case were further concentrated using a rotary evaporator at reduced temperature of 30 ± 1 ^{o}C.

Preparation of stock solutions of the extracts

Stock solution of aqueous and methanolic extracts of *M. villosus* were prepared on each occasion by weighing out 500 mg of the dried extract and subsequent dissolution in 5 ml of dimethylsulfoxide (DMSO) to obtain an extract concentration of 100 mg/ml.

Maintenance, purification and standardization of stock microbial cultures

Stock cultures were maintained on nutrient agar plants at 4^{o}C. Subsequent subcultures were prepared and incubated at 37^{o}C for 18-24 h [15]. A 5 ml volume of sterile distilled water was added to the agar slant containing a 24 h old culture of the desired microorganism and shaken carefully to thoroughly harvest the organisms. Subsequent dilutions were carried out to obtain a microbial population of 1×10^{6} CFU/ml which was the standard inoculum size used in all the tests.

Preliminary antimicrobial screening of the extract

This was done using cup-plate agar diffusion method whereby eight agar plates were seeded with each test microorganism [16-17]. A 0.02 ml volume of the inoculum prepared from the cultures were used. Nutrient agar at a temperature of 40-45^{o}C was poured into plates for bacteria; Sabouraud dextrose agar for fungi and swirled to cool and solidify. Sterile cork borer having a diameter of 8 mm was used to bore holes in the seeded agar plates. Two drops each of a two-fold dilution (100, 50, 25, 12.5 and 3.125 mg/ml) of the extract was added into each labelled hole using a sterile pipette. This was repeated for all the test microorganisms and each performed in three replicates. The plates were incubated at 37^{o}C for 24 h for bacteria and 25^{o}C for 48 h for fungi. The diameters of zones of inhibition produced by the various concentrations of the extract were measured.

Determination of the minimum inhibitory concentration (MIC) of the extracts

From the sensitivity recorded for the antimicrobial screening of the extract, *Staph. aureus*and *B. subtilis*were observed to be most sensitive to the aqueous or methanolic leaf extract of the plant. The MICs of the AE and ME of *M.villosus* were determined separately against the sensitive isolates using the agar-diffusion method.

Four agar plates (each organism in two plates) were seeded with the organism. Graded concentrations were also prepared such that 1 ml volume of the different dilutions of the extracts was introduced into the bored hole in the agar plates with a sterile pipette dropper. The test was done in triplicate using each test isolate after which the plates were incubated at 45^{o}C for 24 h. The inhibitory zone diameters were recorded and determined by plotting a graph of IZD^{2} against the logarithm of the concentrations. The above procedure was also employed to determine the MIC of amoxicillin.

Evaluation of antibacterial effect of the combined extracts and amoxicillin

Stock solution of the extract and amoxicillin were initially prepared to contain varying proportions of extract and amoxicillin ranging from 0:10 to 10:0 according to the continuous variation checkerboard method [17]. Each proportion of the antimicrobial combination was serially diluted with distilled water. A 0.02 ml of the antimicrobial agent was subsequently added into each well on the agar-seeded plates and incubated at 37°C for 24 h. The interactions between the extracts of *M. villosus*and amoxicillin were assessed algebraically by determining their fractional inhibitory concentration (FIC) index according to the following relationships.

(Eqns.1-3):

$$FIC_{index} = FIC_A + FIC_B \ldots\ldots \quad 1$$

where A = *M. villosus*extract, B = amoxicillin.

$$FIC_A = \frac{MIC\ of\ extract\ in\ combination\ with\ amoxicillin}{MIC\ of\ extract\ alone} \quad 2$$

$$FIC_B = \frac{MIC\ of\ amoxicillin\ in\ combination\ with\ extract}{MIC\ of\ amoxicillin\ alone} \quad 3$$

RESULTS AND DISCUSSION

The sensitivity of the test microorganisms to the aqueous and methanolic extracts of *M. villosus*is shown in Table 1. It is evident from Table 1 that the extract had marked activity against Gram-positive organisms (*Staph. aureus* and *B. subtilis*) and no activity against Gram negative bacteria (*K. pneumonia, P. aeruginosa, E. coli and S. paratyphi*) and fungi (*C. albicans*and *A. Niger*). This selectivity in action is suggestive of the fact that the leaf extract of this plant can be used to treat infections caused by *Staph. aureus* and *B. subtilis*. It is however evident that the extract does not possess any antifungal properties. The MICs evaluated from a plot of IZD^2 against the logarithm of concentration for the aqueous and methanolic extracts of *M. villosus* against the susceptible organisms is shown in Table 2 alongside those of amoxicillin. The aqueous extract yielded higher zones of inhibition of the susceptible organisms than the methanolic extract. Water as universal solvent may probably have dissolved much of the biologically active constituents of the leaves, which ultimately produced a greater biological response than the methanolic extract. This is an indication that water is a more effective solvent for the extraction of the antibacterial principles of *M. villosus* than methanol. Thus the differences observed in the activities of the various extracts may also be as a result of the varying degrees of phytoconstituents in the different solvents [18].

Table 1: Sensitivity of the various test isolates to the extracts of *M. villosus*

Test organism	Average Inhibitory zone diameter (mm)	
	Aqueous extract	Methanolic extract.
Klebsiellapneumoniae	-	-
Pseudomonas aeruginosa	-	-
Escherichia coli	-	-
Salmonella paratyphi	16.00	15.50
Bacillus subtilis	22.00	20.00
Staphylococcus aureus	-	-
Candida albicans	-	-
Aspergillusniger		

- = No activity

Table 2: MIC of the extracts and amoxicillin to the test microorganisms

Organism	MIC		
	Aqueous extract (mg/ml)	Methanolic extract. (mg/ml)	Amoxicillin extract. (µg/ml)
Staph. aureus	4.22	4.79	2.80
B. subtilis	5.01	7.59	3.30

Table 3: Antibacterial effect of combined aqueous extract of *M. villosus* and amoxicillin against *Staph.aureus*

Combi-nation ratio	MIC_M:MIC_A*	FIC_M: FIC_A	FIC Index	Activity Index	Effect
10:0	4.22:-	-	-	-	-
9:1	0.4750:0.00004	0.1125:0.013	0.126	-0.900	Synergy
8:2	0.4225:0.00007	0.1000:0.025	0.125	-0.903	
7:3	0.3688:0.00010	0.874:0.036	0.123	-910	
6:4	-	-	-	-	-
5:5	0.2638:0.00018	0.0625:0.063	0.126	-0.900	
4:6	0.2113:0.00021	0.0501:0.076	0.126	-0.900	
3:7	0.1588:0.00025	0.0376:0.089	0.127	-0.896	
2:8	0.105:0.0002	0.0249:0.098	0.123	-0.910	
1:9	0.0528:0.00031	0.0125:0.112	0.112	-0.951	
0.10	-:0.0028				

*M = *Mitracarpusvillosus*; A= Amoxicillin, Activity index = $\log_{10}$ FIC

Table 4: Antibacterial effect of combined aqueous extract of *M. villosus* and amoxicillin against *B. subtilis*

Combi-nation ratio	MIC_M:MIC_A*	FIC_M: FIC_A	FIC Index	Activity Index	Effect
10:0	5:0.10	-	-	-	-
9:1	2.2550:0.00017	0.450:050	0.500	-301	Synergy
8:2	2.0040:0.00033	0.400:0.100	0.500	-0.301	"
7:3	1.540:0.00050	0.350:0.150	0.500	-0.301	"
6:4	1.5030:0.00066	0.300:0.200	0.500	-0.301	"
5:5	0.6250:0.00041	0.130:0.125	0.260	-0.585	"
4:6	0.5010:0.00050	0.100:0.150	0.250	-0.602	"
3:7	0.3760:0.00058	0.075:0.175	0.250	-0.602	"
2:8	0.2505:0.00066	0.05:0.20	0.250	-0.602	"
1:9	0.1250:0.00074	0.025:0.224	0.250	-0.602	"
0.10	-	-:0.0033	-	-	-

Table 5: Antibacterial effect of combined methanolic extract of *M.villosus* and amoxicillin against *Staph.aureus*

Combi-nation ratio	MIC_M:MIC_A*	FIC_M: FIC_A	FIC Index	Activity Index	Effect
10:0	4.79	-	-	-	-
9:1	1.0778:0.0007	0.225:0.025	0.250	-0.602	Synergy
8:2	0.958:0.00014	0.200:0.050	0.250	-0.602	"
7:3	0.838:0.0021	0.175:0.075	0.250	-0.602	"
6:4	0.7185:0.00028	0.150:0.100	0.250	-0.602	"
5:5	0.5988:0.00035	0.125:0.125	0.250	-0.602	"
4:6	0.4790:0.00042	0.100:0.150	0.250	0.602	"
3:7	0.3590:0.00049	0.075:0.175	0.250	-0.602	"
2:8	0.2395:0.00056	0.050:0.200	0.250	0.602	"
1:9	0.1200:0.00063	0.025:0.224	0.250	-0.602	"
0.10	-	-:0.0028	-	-	-

Table 6: Antibacterial effect of combined methanolic extract of *M. villosus* and amoxicillin against *B. subtilis*

Combi-nation ratio	MIC_M:MIC_A*	FIC_M: FIC_A	FIC Index	Activity Index	Effect
10:0	7.59	-	-	-	-
9:1	1.0778:0.0007	0.225:0.025	0.250	0.602	Synergy
8:2	0.958:0.00014	0.200:0.050	0.250	-0.602	"
7:3	-	-	-	-	"
6:4	-	-	-	-	"
5:5	0.9488:0.00041	0.125:0.250	0.250	-0.602	"
4:6	0.7590:9.0005	0.100:0.150	0.250	-	"
3:7	0.5693:0.0006	0.075:0.175	0.250	-	"
2:8	0.3795:0.0066	0.050:0.250	0.250	-	"
1:9	0.1898:0.0007	0.025:0.225	0.250	-	"
0.10	-:0.0033	-:0.0028	-	-	-

The results of the *in vitro* interaction of the extracts in combination with amoxicillin against the susceptible organisms are shown in Tables 3-6.

From the Tables, it is discernible that amoxicillin showed better antibacterial activity that *M. villosus* extracts. The MIC values of the extract were much higher than that of amoxicillin; an indication that a much higher dose of the extract than amoxicillin would be required to elicit the same degree of antibacterial activity as amoxicillin. Moreso, *Staph. aureus* demonstrated greater sensitivity than the extract although the aqueous extract was better in antibacterial activity as against *Staph. aureus* than the methanolic extract. The results of the *in vitro* interaction however showed synergy at all combinations of amoxicillin and aqueous or methanolic extract of *M. villosus* according to the continuous variation checkerboard technique. This observation may be attributed to the probability that the extract of *M. villosus* and amoxicillin, in combination, may act in an additive manner to yield a synergistic antibacterial effect. Synergism is defined as a further decrease in MIC of both agents in combination compared with their individual values [19-20]. Thus, extracts of *M. villosus* and amoxicillin can be used in combination to treat infections caused by *Staph. aureus* or *B. subtilis*.

CONCLUSION

Combination chemotherapy is clinically adopted to achieve a broad-spectrum coverage of invading organisms and to prevent the emergence of resistant organisms. However, clinical application of any combined therapy requires an empirical assessment especially in the event of severe infections. This study has invariably established that use of either aqueous or methanolic extract of *M. villosus* concurrently with amoxicillin would yield greater effectiveness in the treatment of infections in which any of *B. subtilis* or *S. aureus* is implicated than when either of amoxicillin or any of the extracts is used alone.

Further *in vivo* studies would be required to assess the potential usefulness of these preliminary results in real infectious states when any of the organisms showing sensitivity in this study is the invading bacterium.

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