ORIGINAL RESEARCH

IN VITRO ANTIMICROBIAL ACTIVITY OF ETHANOLIC AND METHANOLIC FRUIT EXTRACTS OF *XYLOPIA AETHIOPICA* AND ITS COMBINATION WITH DISC ANTIBIOTICS AGAINST CLINICAL ISOLATES OF BACTERIA AND FUNGI.

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ABSTRACT

Objective: To evaluate the in vitro antimicrobial activity of ethanolic and methanolic fruit extracts of *Xylopia aethiopica* separately and in combination with three antibacterial antibiotics: gentamycin, ofloxacin and ciprofloxacin; and two antifungal antibiotics: fluconazole and ketoconazole. Methods: Clinically isolated strains of bacteria: *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and fungi: *Candida albicans* and *Aspergillus flavus* were used for the assay. The in vitro activities of the methanolic and ethanolic extracts of the *X. aethiopica* plant fruit and the conventional antibiotic discs were individually and initially investigated before the combined evaluation. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were studied. The agar diffusion method was used for the assays. Results: The extracts were active against *P. aeruginosa*, *B. subtilis*, *S. aureus*, *A. flavus* and *C. albicans*. Extracts showed little effect against *K. pneumonia* and no activity against *E. coli*. MIC ranged between 31.25 mg/ml and 125 mg/ml while MBC varied from 15.65 mg/ml to 62.50 mg/ml. There was synergistic interaction between the plant extracts and most of the antibiotics investigated. Conclusion: *X. aethiopica* extract shows antimicrobial activity and therefore the potential for further investigation with a view to finding its relevance in chemotherapy. *X. aethiopica* extract can be administered concurrently with ofloxacin, gentamycin, fluconazole and ketoconazole as against the previously held belief that plant extracts should not be administered together with conventional antibiotics because of apparent antagonism. These findings require in vivo confirmation in animal models.

KEYWORDS: *Xylopia aethiopica*; Disc antibiotics; Plant extract; Antimicrobial; Nigeria; Africa.

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INTRODUCTION

The African environment is probably the least explored in terms of available untapped resources (Nweze et al, 2004). Herbal medicine is readily available in the diverse vegetation and has the potential of possible introduction into modern chemotherapy. Historically, the use of medicinal plants long predates the introduction of antibiotics and other modern drugs to the African continent (Akinyemi et al, 2005; Nweze et al, 2001; Nweze et al, 2005). Herbal medicine has been widely used all over the world and formed an integral part of primary health care in many countries including China (Liu, 1987), Ethiopia (Desta, 1993), and Argentina (Anesini and Perez, 1993). In orthodox medicine, a plant may be subjected to several chemical processes before its active ingredient is extracted, refined and made ready for consumption, while in traditional medicine, a plant is simply eaten raw, cooked, or infused in water or native wine, or prepared as food (Iwu et al, 1999).

A significant proportion of pharmaceutical products in current use are designed from plants (Cowan, 1999). Plants play a two fold role in the development of new drugs; they may become the base for the development of a drug or a natural blueprint for the development of new drugs. It is safe to project that today; plant materials are present in or have provided the models for about 50% of Western drugs (Robbers et al, 1996). The main benefits of using plants derived medicine are that they are often relatively safer than synthetic alternatives and that they provide affordable treatment. Continued and further exploration of plant antimicrobials is needed.

Xylopia aethiopica is a plant which is an integral part of most local foods consumed by people in numerous African countries. This study was carried out to identify the antimicrobial potentials of *X. aethiopica* and to understand whether its concurrent use with conventional antibiotics results in negative interaction.

MATERIALS AND METHODS

Collection of the plant

Fresh *Xylopia aethiopica* fruit were bought from Ogige Market, Nsukka, Nigeria. The plant was identified in the

Department of Botany, University of Nigeria, Nsukka, by a plant taxonomist and a voucher specimen was deposited accordingly.

Processing of the plant

The fresh fruits were washed with tap water and rinsed with sterile distilled water. The material was air dried for several days at room temperature. The dried fruits were pulverized using sterile manual grinder to obtain a powder. This was stored in air tight sterile containers protected from sunlight.

Extraction of active components from the plant

Ethanolic and methanolic solvent extractions were used to extract the active ingredients. Briefly, 50 g each of the powdered fruit was soaked in 450 ml each of the ethanolic and methanolic solvents for 48 hours and then filtered using Whatman's No.1 filter paper and funnel as previously described (Nweze et al, 2004). The filtrates were poured into two sterile plates and evaporated to dryness under air pressure. Both the ethanolic and methanolic dried crude extracts were irradiated with ultraviolet light for four hours and emptied into sterile containers separately and stored at -40 C. The percentage extract yield of powdered material in each solvent was calculated and recorded.

Collection of test organisms

Gram negative bacteria*: P. aeruginosa*, *K. pneumoniae*, *E. coli*, and Gram positive bacteria: *S. aureus* and *B. subtilis*, and fungal organisms: *C. albicans* and *A. flavus* were collected from stock culture in the culture collection of the corresponding author. These bacterial and fungal organisms were reactivated by sub culturing them in a nutrient agar slant (for bacteria isolates) and Sabouraud dextrose agar (SDA) slant (for fungal isolates) using bijou bottles and were incubated as described previously (Nweze et al, 2004).

Reconstitution of the extracts

One gram each of the different extracts was reconstituted with 2 ml each of dimethyl sulfoxide (DMSO) to achieve a concentration of 500 mg/ml each and serially diluted. The test tubes were labeled against the content concentrations.

Preparation of the conventional antifungal discs

The fungal antibiotics fluconazole (FLU) and ketoconazole (KET) were purchased from a pharmaceutical shop in Nsukka, Nigeria. They were pulverized separately using a sterile mortar and pestle to obtain their powdered forms. Five hundred mg was measured from each of the drugs and dissolved separately in 50 ml of distilled water to achieve the concentration of 10 mg/ml solutions of drugs. Using a 6 mm diameter perforator, a Whatman's No.1 filter paper was cut into pieces to obtain the circular discs. Whatman's No.1 filter paper was used because of its high water retention capacity. These discs were put in Petri dishes and oven sterilized at 121ºC for two hours. These sterile discs were soaked in the drug solution for 30 minutes to allow them to absorb the drug solution. These drug impregnated discs were aseptically removed from the solution and kept for use.

Agar diffusion test

The standardized broth form of different organisms were poured in the solid media namely, Mueller Hinton agar for bacteria and SDA for fungi and spread with glass rod to achieve an even distribution of the organisms on the plates. The excess was removed and allowed to dry. With the aid of a sterile standard 6 mm cork borer, 6 wells were bored at equidistant positions. The different concentrations of the extracts were introduced into the different holes and the sixth hole contained the diluent, dimethyl sulfide (DMSO) which was used as the control. This procedure was repeated for all the test organisms and allowed for 30 minutes on the bench and then incubated for 24 hours at 370C. In the second set of culture plates having the same set of organisms, the antibiotic discs were aseptically placed on them and incubated for 24 hours at 370C. The fluconazole and ketoconazole were for fungi and the ciprofloxacin (CPX), gentamycin (CN), ofloxacin (OFX), streptomycin (S), pefloxacin (PEF) and ampicillin (PN) were for the Gram negative and Gram positive bacteria. After 24 hours, the resulting zones of inhibition were measured using a ruler calibrated in millimeters (mm). The average of the 3 readings was taken to be the zones of inhibition of the bacterial or fungal isolate tested at that particular concentration.

Determination of the MIC and MBC

The minimum inhibitory concentrations (MIC) of the extracts were determined by dilution to various concentrations according to the macro broth dilution technique (Nweze et al, 2004; Nweze et al, 2001). Standardized inocula of each organism to be tested was added to series of sterile tubes of nutrient broth containing two fold dilution of the extract and incubated at 37°C for 24 hours. The MIC was read as the least concentration that inhibited the growth of the test organisms. The minimum bactericidal concentration (MBC) was determined by subculturing the test dilution onto fresh drug-free solid medium and incubating further for 18 to 24 hours. The highest dilution that yielded no single cell colony on the solid medium was taken as the minimum bactericidal concentration.

Combined evaluation of the activity of the extracts and antibiotic discs by agar overlay method

The test medium, Mueller Hinton agar (for bacteria) and Sabouraud dextrose agar (for fungi) were prepared, sterilized and allowed to cool. Two ml of the least concentration of the extracts was mixed with the agar and allowed to gel together in the Petri dishes. The Petri dishes containing the media were seeded differently with the different organisms. The excess broth was pipetted off and the cultures were allowed to dry for 30 minutes after which commercially prepared antibiotic discs: gentamycin (CN), ofloxacin (OFX) and ciprofloxacin (CPX) were placed on top of the culture plates and incubated for 24 hours at 37°C. Fluconazole and Ketoconazole discs were prepared in the laboratory and used to test the *C. albicans* and *A. flavus* isolates. The resulting zones of inhibition were measured and the differences in the reaction of the two drugs combined, compared to the antibiotics alone were designated as synergism, indifference or antagonism.

RESULTS

Plant extracts from *X. aethiopica* and antibiotics showed levels of activity on some test isolates while showing no activity on others. The greatest activity of the extracts was observed against the Gram positive bacteria *B. subtilis* and *S. aureus*; while the Gram negative bacterium *E. coli* did not lead to any activity (Tables 1 and 2). Table 3 shows the in vitro activity of the conventional antibiotic against various isolates of bacteria, while Table 4 shows the corresponding values for the fungal isolates. All isolates (bacteria and fungi) were sensitive to the drugs tested. Two of the bacteria isolates, *P. aeruginosa* and *S. aureus*, showed varying resistance to some drugs. For instance, *P. aeruginosa* was resistant to streptomycin and ampicillin but sensitive to all other drugs. *S. auerus* was resistant to pefloxacin, ciprofloxacin and streptomycin.

Table 1: Antimicrobial activity of ethanolic plant extracts from *X. aethiopica* against the isolates of bacteria and fungi.

Organisms	Different extract concentrations 500 mg /ml	250 mg /ml	125 mg /ml	62.5 mg /ml	31.25 mg /ml	MIC (mg/ml)
P. aeruginosa	16	15	14	10	_	62.5 mg/ml
K. pneumonia	15	12	10	_	_	125 mg/ml
E. coli	8	_	_	_	_	500 mg/ml
B. subtilis	18	15	13	13	10	31.25 mg/ml
S. aureus	20	18	16	14	12	31.25 mg/ml
C. albicans	18	17	15	12	10	31.25 mg/ml
A. flavus	19	16	14	13	10	31.25 mg/ml

Key: - no activity

Table 2: Antimicrobial activity of methanolic plant extracts from *X. aethiopica* against the isolates of bacteria and fungi

Organisms	Different extract concentrations 500 mg /ml	250 mg /ml	125 mg /ml	62.5 mg /ml	31.25 mg /ml	MIC (mg/ml)
P. aeruginosa	19	20	17	13	10	31.25 mg/ml
K. pneumonia	19	16	11	_	_	125 mg/ml
E. coli	10	_	_	_	_	500 mg/ml
B. subtilis	17	20	22	17	14	31.25 mg/ml
S. aureus	22	17	16	15	13	31.25 mg/ml
C. albicans	20	19	17	15	13	31.25 mg/ml
A. flavus	24	18	15	13	11	31.25 mg/ml

Key: - no activity

Table 3: Inhibition zone diameter (mm) of the antibiotic disc against the bacterial isolates.

Organisms	Ofloxacin	Pefloxacin	Ciprofloxacin	Streptomycin	Ampicillin	Gentamycin
P. aeruginosa	20	20	25	_	_	19
K. pneumoniae	40	28	20	18	15	20
E. coli	_	18	11	16	14	13
B. subtilis	20	23	25	22	18	20
S. aureus	14	_	_	_	14	15

Key: - no activity

Table 4: Inhibition zone diameter (mm) of the antifungal antibiotic disc against fungi isolates.

Fungal isolates	Fluconazole	Ketoconazole
C. albicans	18	15
A. flavus	20	16

Table 5 shows the in vitro combined activities of the plant extract from *X. aethiopica* and the disc antibacterial antibiotic, while Table 6 shows the corresponding values for the combination of the plant extract and the two antifungal drugs evaluated. Tables 7 and 8 show the summary of the combinations of the ethanolic and methanolic plant extracts from *X. aethiopica* and the conventional antibiotics against all the isolates, respectively. For both the ethanolic and methanolic extract combinations with the antibiotics, synergistic interaction was obtained in 68.4% of the combinations investigated, while antagonism was observed in 26.3%, and indifference in 5.3%.

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Table 5: Inhibition zone diameter (mm) of the combined activity of the plant extracts from *X. aethiopica* and the antibiotic discs against bacterial isolates.

	Ethanolic extract with antibiotic			Methanolic extract with antibiotic		
Bacterial isolates	Ofloxacin	Ciprofloxacin	Gentamycin	Ofloxacin	Ciprofloxacin	Gentamycin
P. aeruginosa	25	16	36	22	11	28
K. pneumonia	16	8	18	17	10	20
E. coli	20	17	23	19	10	18
B. subtilis	28	20	20	22	19	29
S. aureus	29	10	28	35	14	30

Table 6: Inhibition zone diameter (mm) of the combined activity of the plant extracts from *X. aethiopica* and the antibiotic against fungal isolates.

	Ethanolic extract with antibiotic		Methanolic extract with antibiotic	
Organisms	Fluconazole	Ketoconazole	Fluconazole	Ketoconazole
C. albicans	23	26	25	28
A. flavus	30	28	32	28

Table 7: Summary of the ethanolic plant extract from *X. aethiopica* and antibiotic combination against all isolates.

Test organisms	Antibiotic disc	IZD* of antibiotic disc (mm)	IZD of the disc/extract (mm)	% Variation	Result of the combination
P. aeruginosa	Ofloxacin	20	25	25	Synergism
	Ciprofloxacin	25	16	-36	Antagonism
	Gentamycin	19	36	89.5	Synergism
K. pneumoniae	Ofloxacin	40	16	-60	Antagonism
	Ciprofloxacin	20	8	-60	Antagonism
	Gentamycin	20	18	-10	Antagonism
E. coli	Ofloxacin	16	20	25	Synergism
	Ciprofloxacin	11	17	54.5	Synergism
	Gentamycin	13	23	76.9	Synergism
B. subtilis	Ofloxacin	20	28	40	Synergism
	Ciprofloxacin	25	20	-20	Antagonism
	Gentamycin	20	20	0	Indifference
S. aureus	Ofloxacin	14	29	107	Synergism
	Ciprofloxacin	-	10	-	Synergism
	Gentamycin	15	20	33.3	Synergism
C. albicans	Fluconazole	18	23	27.8	Synergism
	Ketoconazole	15	26	73.3	Synergism
A. flavus	Fluconazole	20	30	50	Synergism
	Ketoconazole	16	28	75	Synergism

*IZD = Inhibition zone diameter (mm)

Table 8: Summary of the methanolic plant extract from *X. aethiopica* and antibiotic combination against all isolates.

Test organisms	Antibiotic disc	IZD* of antibiotic disc (mm)	IZD of the disc/extract (mm)	% Variation	Result of the combination
P. aeruginosa	Ofloxacin	20	22	10	Synergism
	Ciprofloxacin	25	11	-56	Antagonism
	Gentamycin	19	28	47.4	Synergism
K. pneumonia	Ofloxacin	40	17	-57.5	Antagonism
	Ciprofloxacin	20	10	-50	Antagonism
	Gentamycin	20	20	0	Indifference
E. coli	Ofloxacin	16	19	18.8	Synergism
	Ciprofloxacin	11	10	-9	Antagonism
	Gentamycin	13	18	38.5	Synergism
B. subtilis	Ofloxacin	20	22	10	Synergism
	Ciprofloxacin	25	19	-24	Antagonism
	Gentamycin	20	29	45	Synergism
S .aureus	Ofloxacin	14	35	150	Synergism
	Ciprofloxacin	-	14	-	Synergism
	Gentamycin	15	30	100	Synergism
C. albicans	Fluconazole	18	25	38.9	Synergism
	Ketoconazole	15	28	86.7	Synergism
A. flavus	Fluconazole	20	32	60	Synergism
	Ketoconazole	16	28	75	Synergism

*IZD = Inhibition zone diameter (mm)

DISCUSSION

The results of the antimicrobial activity of the ethanolic and methanolic extracts of *X. aethiopica*, suggest that the plant extracts were very active against some of the test organisms such as *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *A. flavus*. However, they showed little activity against *K. pneumonia* and had no observable activity against *E. coli*. The lack of activity against *E. coli* may be due to the fact that *E. coli*, a Gram negative bacterium, has an extra outer membrane that may be impermeable to the plant extract. This observation is similar to the findings of Iwu (1993) and Boakiji-Yiadom et al. (1977). Some authors (Konning et al., 2004; Tatsadjieu et al., 2003; Asekun and Adeniyi, 2004) have reported in vitro activities of this extract against other microorganisms including *E.coli* and *K. pneumonia* contrary to some of our findings in this study. This is not unexpected considering that specific strain differences and previous exposure of these isolates to antibiotics are important factors which can influence the outcome of susceptibility tests. It is advisable to test a large collection of isolates before drawing conclusions. In the above studies only one isolate of each organism was tested.

The differences in the spectrum of activities of the extracts show the concentrations at which the extracts have the best antimicrobial activities. These results appear to correlate with the claims of some herbalists that extracts from *X. aethiopica* have medicinal and antimicrobial uses and potentials. From the results, it can be deduced that the antimicrobial activity of the extracts declined with decreasing concentrations in the majority of the cases. However, there were exceptions. For instance, against *B. subtilis*, the inhibition zone diameter achieved with the highest concentration of 500 mg/ml was 17 mm but the second and third lower concentrations, 250 mg/ml and 125 mg/ml, had inhibition zone diameters of 20 mm and 22 mm, respectively. Similar observations were noticed against *P. aeruginosa*. The reasons for these results are unknown.

Some of the conventional antibiotics had inhibitory effects on the isolates except for *P. aeruginosa*, which showed resistance to Streptomycin and Ampicillin and *S. aureus*, which showed resistance to Pefloxacin, Ciprofloxacin and Streptomycin. Ofloxacin produced the highest zone of inhibition of 40 mm against *K. pneumonia*, while Ciprofloxacin produced the least inhibition zone diameter of 11 mm on *E. coli*. The reason for these differences could be due to the fact that in Nigeria Ciprofloxacin is more often used than Ofloxacin.

In the drug/extract combination tests some interesting findings were observed. For example, *E. coli* which was resistant to both the plant extracts and the conventional antibiotic, Ciprofloxacin alone, became susceptible to the combination of the two. It is likely that each drug has a different mechanism and may have attacked *E. coli* from different angles, making the organism more susceptible. While the inhibition zone diameters obtained against some of the organisms such as *P. aeruginosa*, *C. albicans*, *A. flavus*, and *S. aureus* increased, others such as *K. pneumonia* reduced, while *E. coli* and *B. subtilis* remained indifferent. This may be attributed to the synergistic or antagonistic interaction of the two drugs. The methanolic extract in combination with Ofloxacin had the highest percentage variation of synergism of 150% against *S. aureus*. When combined with Gentamycin, it showed a synergistic percentage variation of 100% against *S. aureus*. Similarly, against *C. albicans* and *A. flavus*, the methanolic extract combined with Ketoconazole showed synergy when compared to the activity of the antibiotics alone.

The MIC and MBC results of the two extracts indicated that the extracts were bactericidal to *K. pneumonia* and *B. subtilis*. This correlates with the recent work of Umeh and co-workers (2005) who documented the inhibitory effects of methanolic extract of some plants from Benue State, Nigeria. Although both the ethanolic and methanolic extracts showed good activities, the later was better because it showed more pronounced synergistic association with the antibiotics. It has been previously argued that methanolic extracts are better since methanol extracts both polar and non-polar compounds (Okafor et al, 2001). Our results, however, also justify the use of *X. aethiopica* extract with ethanol.

Results of the present in vitro study suggest that the concurrent administration of the plant extract from *X. aethiopica* with any of the conventional antibiotics may not necessarily elicit antagonisms as has been a widespread belief. However, in vivo studies will be required for the eventual confirmation of our observations.

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