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EVALUATION OF THE ANTIMICROBIAL PROPERTIES OF DIFFERENT PARTS OF *CITRUS AURANTIFOLIA* (LIME FRUIT) AS USED LOCALLY

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Abstract

We investigated the potency of *Citrus aurantifolia* (Lime fruit), against pathogens, in the different forms in which this fruit plant is used locally (juice of the fruit, burnt rind of the fruit commonly known as "epa-ijebu" in the Yoruba dialect) and the oil obtained from steam distillation of the fruit. The antimicrobial activity of "epa-ijebu" in different solvents was also compared. The solvents include palm-wine (a local alcoholic drink tapped from palm trees), Seaman's Schnapps 40% alcoholic drink, water, ethanol and fermented water from 3 days soaked milled maize known as "ekan-ogi" or "omidun" in the Yoruba dialect. Antimicrobial activity was carried out by the agar well diffusion. The clinical isolates used included Anaerobic facultative bacteria, namely: *Staphylococcus aureus* ATCC 25213, *Staphylococcus aureus, Salmonella paratyphi, Shigella flexnerii, Streptococcus faecalis, Citrobacter spp, Serratia spp, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli ATCC 25922,* and *Escherichia coli;* Fungi such as *Aspergilus niger* and *Candida albicans; and* Anaerobes which includes *Bacteroides* spp, *Porphyromonas spp,* and *Clostridium spp.* Crude extracts of all solvents used varied in zones of inhibition. The anaerobes and the Gram-positive bacteria were susceptible to all the extracts with minimum inhibitory concentration (MIC) ranging from 32mg/ml-128g/ml. The activity against the fungi showed only the oil extract potent for *A. niger*, while *Candida albicans* was susceptible to all the extracts with MIC ranging from 256mg/ml-512mg/ml. The Gram-negatives have MIC ranging from 64mg/ml-512mg/ml. Minimum bactericidal concentration (MBC) ranged between 32mg/ml to 512mg/ml depending on isolates and extracting solvent. The oil and palm-wine extract of "epa-ijebu" showed greater activity than the other extracts. The killing rate of the schnapps extract on *S. aureus* and *E. coli* was 1 and 3.5 hours respectively.

Introduction

Traditionally, usage of plants in curing illness has deep roots in man's history (Grabley and Thiericke, 1999). Ethno pharmacological use of plants prevails among Nigerian native people. Plants are used in treating malaria, diarrhoea, burns, gonorrhoea, stomach disorders and other infectious diseases. Efforts of scientists in establishing plants with promising antimicrobial activity are yielding fruitful results (Ndukwe et al., 2005; Adedayo et al., 2001; Perumal and Ignacimuthu 2001; Oyagade et al., 1999; Olukoya et al., 1986). The plants are easily available and cheaper than the conventional drugs. One of such plants is *Citrus aurantifolia* (Lime fruit). Lime in its natural state is widely used in West Africa, particularly in Nigeria. From information gathered from market study in the southwest part of Nigeria, lime is very much employed in herbal medicine. It is an essential ingredient in the preparation of most herbal concoction; it is also used to suppress stomachache. When added to sugar and palm oil or to honey, the juice has been found to be an excellent cough relieving mixture. The rind is burnt in some homes to act as insecticides against mosquitoes. The mesocarp is also used as a very good facial scrub and helps in

prevention of pimples due to its cleansing action on the skin. Literature exists on the antimicrobial activities of lime in conjuction with other extracts (Onyeagba *et al*., 2004). Given the alarming incidence of antibiotic resistance in bacteria of medical importance, (Aibinu *et al*., 2003a; Aibinu et al., 2003b; Aibinu et al., 2004a; Aibinu et al., 2004b); there is a constant need for new and effective therapeutic agents. This study is part of an ongoing project to search for novel drugs from a vast array of medicinal plants from the southwest part of Nigeria. The aim of this study is to investigate the potency of lime, in the different forms in which it is used locally, against pathogens, and to compare its activity in the different solvents used in traditional medicine.

Materials and Methods

Collection and Preparation of Plant Extract

The lime fruits (unripe) were purchased from Mushin market in Lagos-State, Nigeria. The fruits were identified at the Department of Pharmacognosy, College of Medicine, University of Lagos, Nigeria.

Extract A: 50 lime fruits were cut into smaller pieces in a tray. This was kept in the oven at 50^0C for a whole week until the whole fruits were completely dried and burnt (This is another traditional way of using lime fruit.This burnt lime fruit is locally known as 'epa-ijebu' and it is known in Yoruba dialect as **"**gbogbo nise**"** to be an 'all disease healing herbal medicine'). The dried and burnt fruit was grounded into fine powder. Twenty-five grams of the powder was weighed and put in 5 different sterile universal bottles and labelled A1-A5. They were kept at 30 ^{o}C (room temperature).

Extract B: A second set of 20 lime fruits was aseptically squeezed into sterile bottles to obtain its juice and kept at 4 ^{o}-8 ^{o}C.

Extract C: A third set of 80 lime fruits was used in obtaining the Lime fruit oil by steam distillation of rind of whole fruit. (This was carried out at the Department of Pharmacognosy, College of Medicine, University of Lagos, Nigeria.).

Concentration and Preparation of Stock Solutions

Five different solvents namely: (i) distilled water, (ii) ethanol, (iii) palm-wine (emu), (iv) Seamann's Schnapps ® (Nigeria Distilleries Limited, Sango-Ota; 40 % alcohol) and (v) fermented water from 3 days soaked grounded maize/corn paste (ekan-ogi/omi-ogi) were used for extraction of extract A. Twenty-five grams of extract A was weighed and soaked in each of the 5 solvents. This was left to soak at room temperature for 3 days with agitation at intervals. The extracts from each solvent was decanted, passed through muslin cloth, and then filtered with Whatman No. 1 filter paper. All extracts were tested for purity by plating them on nutrient agar and incubated for 24 hours at 37 ^{o}C and 25 ^{o}C. The extracts from each solvent were taken for lyophilization at the Department of Biochemistry, College of Medicine, University of Lagos. Each solid extract/paste obtained after lyophilization was reconstituted in their respective solvents to obtain a stock solution of 512 mg/ml according to the National Committee for Clinical Laboratory Standards (NCCLS/CLSI) (2000). The stock solutions were stored in sterile capped bottles and kept at 4 ^{o}C-8 ^{o}C.

Test organisms

The organisms used comprise of seven gram-negative organisms (*Serratia* spp, *Salmonella paratyphi*, *Shigella flexnerri*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Citrobacter* spp, and *Escherichia coli*,); two gram-positive organisms (*S. aureus,and Enterococcus feacalis*),two fungi (*Aspergillus niger* , and *Candida albicans*) and three anaerobes (*Clostridium* spp, *Bacteroides* spp and *Porphyromonas* spp).The test organism were obtained from the research laboratory of Medical Microbiology and Parasitology of college of Medicine ,University of Lagos.

Control Organisms

Control strains of *Staphylococcus aureus* ATTC 25921 and *Escherichia coli* ATTC 25992 were used and tested along with the organisms.

Standardisation of inoculum

Test organism were subcultured onto fresh plates of nutrient agar (Oxoid, UK) for 24 hrs and Saboraud dextrose agar (Oxoid, UK) for 5-7 days at 37 º C for bacteria and fungi respectively. Anaerobes were subcultured onto blood agar and incubated for 72 h in an anaerobic jar at 37 º C. Colonies from these plates were suspended in Mueller-Hinton broth (Oxoid, UK), Sabouraud broth (Oxoid, UK) and Wilkin Chalgrens broth (Oxoid, UK) to a turbidity matching 0.5 mc McFarland standard (10^8 cfu/ml) for bacteria, fungi and anaerobes respectively.

Media used for antimicrobial assays are Mueller-Hinton agar (Oxoid, UK) for bacteria and Sabouraud agar (Oxoid, UK) for fungi and Wilkins Chalgren's agar (Oxoid, UK) for anaerobe. All were incubated appropriately as specified for each organism.

Antimicrobial Assay

Suspension of the microorganisms were made in sterile normal saline and adjusted to 0.5 McFarland standards. The resulting suspension contains approximately 1 X 10^8 cfu/ml. Each labelled medium plate was uniformly seeded with a test organism by means of sterile swab rolled in the suspension and streaked on the plate surface. Wells of 5 mm in diameter and about 2 cm apart were punched in the culture media with sterile cork borer. The various concentrations were dropped into each well to fullness (Shahidi, 2004). This is approximately 100 µl. The oil extract was used at neat and at 50%v/v in methanol. Each plate was kept in the refrigerator at 4 ^oC for 1 h before incubating at 37 ^oC for 24 h. Zones of inhibition around the wells, measured in millimetres, were used as positive bioactivity.

Controls

Ciprofloxacin antibiotic suspension (1:19 dilution) and neat solvents without test compounds were placed in wells on each plate along with the test extracts.

Determination of minimum inhibitory concentrations (MICs)

The MIC was defined as the lowest concentration able to inhibit any visible bacterial growth on the culture plates (Shahidi, 2004; Prescott *et al.*, 1999). This was determined from readings on the culture plates after incubation.

Determination of the Minimum Bactericidal Concentration (MBC) and Minimum fungicidal concentration (MFC)

To a 0.5 ml extract at different concentration of doubling diffusion as used in MIC determination was added 0.5 ml of test organism in tubes. These were incubated at 37 ^oc for 24h, 72 h and 5-7 days for bacteria and fungi respectively. Samples were streaked out from the tubes on to Nutrient agar, Sabouraud agar (Oxoid, UK) and blood agar to determine the minimum concentration of the extract required to kill the organisms. These concentrations were indicated by failure of the organism to grow on transfer to these media plates. The lowest concentration that prevented bacterial/fungal growth after days of incubation was recorded as the minimum bactericidal/fungicidal concentration (MBC/MFC).

Determination of death rate of isolates

Death rate of the most susceptible and the least susceptible bacteria used (*S.aureus* and *Escherichia coli*) was carried out. This was done by mixing 0.1ml of 10^8 CFU/ML of the test organism with 0.9 ml of stock (512 ug/ml) of the schnapps extract. 0.1 ml of the mixture was taken and plated out on sterile nutrient agar at 30 minutes interval for 10 times 1.e (30 min –300min). They were incubated at 37 ^oC for 24 h. The number of colonies on each plate at the time intervals was counted.

Results

The result showed that all the extracts at the stock concentration have antimicrobial activity. Under the conditions employed here, all test samples have potent inhibitory effects on the groups of bacteria and fungi tested (Tables 1 and 2). The lime oil (neat) showed a high antimicrobial activity against all organisms tested with zone diameters ranging from 13 mm (*Pseudomonas aeruginosa*) to 48mm (*Aspergillus niger*). Highest activity of the oil was observed against the fungi with zone diameters of 40 mm and 48 mm for *Candida* spp and *Aspergillus niger* respectively. None of the other extracts had activity against *A. niger* except the oil. While for *Candida albicans*, all extracts had antifungal activity against it. For *Shigella flexnerii*, the water based extracts (omi-ogi and the aqueous extract) with the limejuice were observed to be more effective than other extracts

Apart from the oil, the palmwine extract had the highest activity against both gram-positive and gram-negative isolates and this was closely followed by the Seamann's schnapps extract ® and ethanol extract with the aqueous extract and lime juice following. Omi-ogi extract had the least activity against these isolates. The anaerobes were highly susceptible to all the extracts with zone diameter ranging from 26 mm (omi-ogi extract) to 40 mm (lime oil). *Staphylococcus aureus* was the most susceptible isolate tested while *Escherichia coli* was the least susceptible isolate. The MIC and MBC ranged between 32 mg/ml to 512 mg/ml depending on isolates and extracting solvent. Crude extracts of all solvents used varied in zones of inhibition. Schnapps solvent did not inhibit any of the isolates when used alone but was potent against the organisms when used with the extract; omi-ogi solvent inhibited all the gram-positive isolates and a few of the gram-negatives when used alone but was the least potent when used in combination with the extract. The palm-wine as a solvent inhibited only *Serratia* spp, *Salmonella paratyphi* and *Ps. aeruginosa* while in combination with the extract was highly potent against all organisms except *A. niger*. The ethanol solvent (75 %) inhibited most of the organisms. The killing rate of the schnapps extract on *Staphylococcus aureus* and *Escherichia coli* was 1 h and 3.5 h respectively. Time course study of Schnapps' extract on *Staphylococcus aureus*, showed the extract was able to kill all the cells after 1 h of exposure. The killing effect of the extract on *E. coli* was gradual in the first 2.5 h of exposure but became drastic 30 min later and the surviving cells were reduced to zero after 3.5 h of exposure.

Table 1: Minimum Inhibitory Concentraton (MIC) of Isolates to *Citrus aurantifolia* (Lime fruit) Extracts

	Concentrations (mg/ml)					
Test Organisms	PWE	SE	EE	AQE	JUICE	OOE
Gram-Positive Organisms						
Staphylococcus aureus ATCC 25213	32	64	64	256	256	128
Staphylococcus aureus (1)	32	32	64	128	64	128
Enterococcus faecalis	128	64	256	256	256	128
Gram-Negative Organisms						
Escherichia coli ATCC 25922	128	64	256	256	256	256
Salmonella paratyphi	64	256	256	256	256	256
Citrobacter spp	512	128	256	256	256	256
Serratia spp	64	512	64	256	128	256
Shigella flexnerii	256	256	256	256	64	128
Klebsiella pneumoniae	256	256	256	256	128	128
Pseudomonas aeruginosa	128	128	256	256	256	128
Escherichia coli	128	64	128	256	256	256
Fungi						
Candida albicans	256	256	256	512	256	512
Aspergilus niger	-	-	-	-	-	-
Anaerobes						
Bacteroides spp	64	128	128	128	128	128
Clostridium spp	32	32	64	64	128	128
Porphyromonas spp	32	32	64	64	128	128

KEY:
SE=Schnapps extract; Juice=Lime juice; PWE=Palm wine extract; EE=Ethanol extract
AQE=Aqueous extract; OOE=Omi-ogi extract

Table 2: Zones of inhibition (mm) of organisms to *Citrus aurantifolia* (Lime fruit) Extracts at Neat and Stock Concentration of 512mg/ml

Test Organisms	CIP	OIL.E (Neat)	PWE	SE	EE	AQE	JUICE (Neat)	OOE
Gram-Positive Organisms								
Staphylococcus aureus ATCC 25921	20	30	26	24	22	22	24	24
Staphylococcus aureus (1)	20	40	30	28	35	33	22	25
Enterococcus faecalis	16	25	26	22	20	24	20	20
Gram-Negative Organisms								
Escherichia coli ATCC 25922	27	30	19	25	14	16	17	19
Escherichia coli	27	25	20	20	15	20	14	15
Shigella flexnerii	33	30	19	19	17	21	21	20
Salmonella paratyphi	30	40	17	19	12	21	9	18
Citrobacter spp	30	19	17	18	16	22	12	18
Serratia spp	25	17	24	21	24	17	13	23
Pseudomonas aeruginosa	17	13	18	24	20	19	14	15
Klebsiella pneumoniae	30	19	16	18	16	15	17	17
Fungi								
Aspergilus niger	-	48	40	0	0	0	0	0
Candida albicans	-	40	30	30	30	15	32	18
Anaerobes								
Porphyromonas spp	26	27	23	22	18	18	21	15
Clostridium spp	27	31	28	25	23	20	22	15
Bacteroides spp	24	30	21	21	20	18	20	18

KEY: Oil .E=Oil extract; SE=Schnapps extract; PWE=Palm wine extract; EE=Ethanol-extract; AQE=Aqueous extract ; OOE=Omi-ogi extract Juice=Lime juice CIP=Ciprofloxaci**n**

Discussion

Investigation of the antimicrobial activity of limejuice alone and in combination with other herbs has been investigated (Rodriguez *et al*., 2000; Onyeagba *et al*., 2004)) and limejuice has been found to have high antimicrobial activity. In this study it is observed that the potency of lime fruit is enhanced by the type of solvent used indicating that there are some active ingredients in lime which have high antimicrobial/antifungal effect but which would not be released except when lime fruit is used in conjunction with a particular solvent. This is in agreement with a report by Taylor (2004), which states that water is almost universally the solvent used to prepare herbs traditionally. In some cases, however, some active components found in plants are not soluble in water, therefore just preparing a hot tea with the plant or even boiling the plant in hot water, won't extract these active components. This is the reason why some plants are prepared in one manner to treat one specific condition, yet are prepared in a different way to treat a completely different condition. Preparing an infusion/tea of a plant might extract a delicate group of anti-inflammatory plant steroids to treat arthritis. Yet when the same plant is prepared in alcohol as a tincture, different antibacterial alkaloids are extracted instead (Taylor, 2004). In this study, limejuice known to be very potent in treatment of infectious diseases and ailments when used alone and in combination with other herbs, ranks fifth in antimicrobial potency when compared with the other forms and type of solvent in which lime fruit is used locally. This implies that there are still lot more to gain from lime fruit as an antimicrobial agent when used in the other forms and in solvents such as Schnapps ® and palm-wine. The result of this study is a pointer to new sources of novel drugs, which needs to be further investigated. Many plants are used in Nigeria in the form of crude extracts, infusions or plasters to treat common infections without any scientific evidence of efficacy. In this study, the microbiological investigation done on lime fruit obtained in Nigeria have shown activity coherent with the use of this plant in folk medicine.

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