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IN VITRO ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACTS FROM PLANTS
BRYOPHYLLUM PINNATUM AND *KALANCHOE CRENATA*

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Abstract

Extracts from the leaves of *Bryophyllum pinnatum* and *Kalanchoe crenata* were screened for their antimicrobial activities. Solvents used included water, methanol, and local solvents such as palmwine, local gin (Seaman's Schnapps 40% alcoholic drink,) and "omi ekan-ogi" (Sour water from 3 days fermented milled maize). Leaves were dried and powdered before being soaked in solvents for 3 days. Another traditional method of extraction by squeezing raw juice from the leaves was also employed. All extracts were lyophilized. These extracts were tested against some Gram-negative organisms (*Escherichia coli* ATCC 25922, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Shigella flexneri*, *Salmonella paratyphi*, *Citrobacter spp*); Gram-positive organisms *Staphylococcus aureus* ATCC 25213, *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*) and a fungus (*Candida albicans*). Agar well diffusion and broth dilution methods were used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) at concentrations of 512mg/ml to 4mg/ml. All the organisms except *Candida albicans* were susceptible to the extracts obtained from the traditional method. The squeezed-leaf juice of *Kalanchoe crenata* was the most active one with MIC of 8 mg/ml against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Bacillus subtilis*, 32 mg/ml against *Shigella flexneri*, 64 mg/ml against *Escherichia coli* and 128 mg/ml against the control strain *Staphylococcus aureus* while its MBC is 256 mg/ml against these organisms except *Bacillus subtilis* and *Klebsiella pneumoniae*. The Gram-positive organisms were more sensitive to the methanol and local gin-extract of *Bryophyllum pinnatum*. Extracts from other solvents showed moderate to weak activity.

Key words: Antimicrobial, *Bryophyllum pinnatum*, *Kalanchoe crenata*, Local solvents, Gram-positive organism, Gram-negative organism

Introduction

In recent times, there have been increases in antibiotic resistant strains of clinically important pathogens, which have led to the emergence of new bacterial strains that are multi-resistant (WHO, 2001, Aibinu et al., 2003; Aibinu et al., 2004). The non-availability and high cost of new generation antibiotics with limited effective span have resulted in increase in morbidity and mortality (Williams, 2000). Therefore, there is a need to look for substances from other sources with proven antimicrobial activity. Consequently, this has led to the search for more effective antimicrobial agents among materials of plant origin, with the aim of discovering potentially useful active ingredients that can serve as source and template for the synthesis of new antimicrobial drugs (Pretorius et al., 2003, Moreillon et al., 2005).

Bryophyllum pinnatum and *Kalanchoe crenata* belong to the plant family Crassulaceae. They show close proximity in usage, habitat, preparation and identification. The external morphological features of *Kalanchoe crenata* resemble that of *Bryophyllum pinnatum*. An inexperienced taxonomist and even unseasoned traditional practitioner can readily confuse the two plants. This is attested to by the same local name that is being used by Yorubas in Southwest Nigeria. Some People refer to both plants as “Odundun”. Ethnobotanically, most often they are prepared and administered the same way. *Bryophyllum pinnatum* is used in ethnomedicine generally for the treatment of ear-ache, cough, diarrhoea, dysentery, abscesses, ulcers, insect bites, heart-troubles, epilepsy, arthritis, dysmenorrhoea and whitlow (Gill, 1992). In southern Nigeria, it is used to facilitate the dropping and healing placenta wound of newly born babies. The plant leaf is mildly exposed to heat and the juice is squeezed out and applied as poultice to the baby’s placenta on daily basis. Also, the crushed leaves as well as the extracted juice are mixed with shear butter or palm oil and rubbed on abscesses or other swellings. This is also applied on ulcers, burns and on the bodies of young children when they are ill. The leaves of this plant contain bryophyllin, potassium, malate, ascorbic, malic, and citric acids (Oliver, 1989). The plant is rich in both macro and micro elements, vitamins, calcium, phosphorus, ascorbic acid, inulin (Okwu and Josiah, 2006) and other compounds like saponin, flavonoids, anthraquinones, xanthenes, bryophyllin A and B (Iwu, 1993). Anti-inflammatory, hypoglycaemic, anti diabetic and anticancer properties have been reported (Gill, 1992).

The external applications of *Kalanchoe crenata* are the same as those of *Bryophyllum pinnatum*. The juice obtained by squeezing the leaves that have been passed over fire slightly, is most commonly used for the treatment of headache, general debility, dysentery, smallpox and convulsion. One or two drops of the leaf juice is dropped into the ear for earache. A poultice of the leaves is applied over wounds and sores. The leaves can be boiled in water and the extract is given as a sedative for asthma and palpitation. Also the leaf juice mixed with salt and honey is a remedy for chronic cough. The extract of dried leaves is applied to septic wound (Sofowora, 1993).

In East Africa, the slightly heated leaves (heated over fire) are rubbed over the body as treatment for stiff joint and rheumatism (Gill, 1992). Other parts of the plant especially the root is prescribed for gonorrhoea, vermifuge and abortion (Sofowora, 1993). Alkaloids and saponins are present in the aqueous and alcoholic extracts of leaves and lectins in the juice from the fresh leaves (Nguelefack et al., 2006). The green callus of the plant contain malic acid, quinones and tocopherol (Sofowora, 1993; Oliver, 1989).

Other works have also shown that this plant possesses analgesic, anticonvulsion, antiinflammatory, antiarthritic and antispasmodic properties (Theophil et al., 2006). The conventional method to extract plant materials is to use methanol, ethanol, acetone and so on as extracting solvents, but the ethnobotanical approach like the use of Palmwine, “Omidun/ekan-ogi”(the water derived from three days fermented milled maize), local gin as extracting solvent and ways in which they are prepared locally, has received less attention. The type of solvents and methods of preparation affect antimicrobial activity of plants (Ellof, 1998; Adesanya, 2005). On the basis of this background, in-vitro antimicrobial activities of the extracts *Bryophyllum pinnatum* and *Kalanchoe crenata* from various solvents were tested against clinically important pathogens.

Materials and Method

Plant Materials

The leaves of *Bryophyllum pinnatum* and *Kalanchoe crenata* were collected from Oke-igbo in Ondo State. These plants can be collected during rainy season particularly between March and July. The samples were identified by Mr. T.K., Odewo of the Herbarium Department, Forestry Research Institute of Nigeria (FRIN) at Ibadan, Oyo State. The leaves were dried in an oven at 45°C for fourteen days. The dried leaves were powdered and stored in a sterile bottle at room temperature (Shahidi Bonjar, 2004). Both aqueous and methanol extraction were carried out by using Soxhlet extractor (Quickfit U.K). Powdered dried leaves (50 g) were extracted with 250 ml of solvent (Akinyemi et al., 2005).

Traditional methods of extraction includes:

Steeping.

The powdered leaves (50 g) were suspended in the following solvents – Palm-wine, “Omidun” and the local gin (Seaman’s Schnapp from Nigeria Distilleries Ltd), inside a conical flask. The conical flasks were shaken intermittently for three days at room temperature. The resulting extracts were decanted, filtered using Muslin cloth and Whatman No 1 filter paper and stored in sterile bottles (Atata, 2003).

Squeezing of the leaf part

Fresh leaves (300g) were passed over heat using the bursen burner; similar to the way they are prepared traditionally. The leaves were then squeezed and the resulting juice was filtered using a sterile Muslin cloth and Whatman No 1 filter paper and stored in sterile bottle. Extracts from all these extraction methods were tested for purity by plating them on Mueller Hinton Agar (MHA) (Oxoid, UK) and incubated for 24 hours at 37°C. All the resulting extracts were lyophilized in a freeze dryer (Edwards, USA). The powdered forms were weighed, labeled, stored in sterile bottles and kept in refrigerator.

Preparation of Test Samples

In the study of the antimicrobial activities of these plants, concentrations of 512 mg/ml of each extract were used for the screening. This was done by dissolving 2.1 g of the extracts in 4 ml of each of the extracting solvents. For the squeezed juice that was lyophilized, distilled water was used to suspend the sample. Each of the solid extracts was reconstituted in its respective solvent to obtain a stock solution of 512 mg/ml (NCCLS, 2000).

Sources and maintenance of organisms

Gram-positive organisms (*Staphylococcus aureus* ATCC 25213, *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*), Gram-Negative organisms (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* ATCC 25922, *Escherichia coli*, *Shigella flexneri*, *Salmonella paratyphi*, *Citrobacter species*) and a fungal yeast *Candida albicans* were obtained and confirmed at the research laboratory of the Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos. They were maintained on Mueller-Hinton Agar medium (Oxoid, UK). Twenty-four hour old pure cultures were prepared for use each time.

Culture media

Mueller-Hinton Agar (Oxoid, UK) was prepared according to the manufacturer's instruction, autoclaved and dispensed at 20 ml per plate in 12 x 12cm Petri dishes. Set plates were incubated overnight to ensure sterility before use.

Antimicrobial Bioassay

Suspension of micro-organisms was made in sterile normal saline and adjusted to 0.5 Macfarland standard (10^8 Cfu/ml) (NCCLS, 2000). From the stock of 512mg/ml extract, serial dilutions were made to 256, 128, 64, 32, 16, 8, 4 mg/ml (NCCLS, 2000). Each labelled medium plate was uniformly inoculated with a test organism by using a sterile cotton swab rolled in the suspension to streak the plate surface in a form that lawn growth can be observed. A sterile cork borer of 5mm diameter was used to make wells on the medium. 0.1ml of the various extract concentration were dropped into each, appropriate labeled well (Atata 2003; Shahidi Bonjar 2004). Other solvents used for extraction apart from water were tested neat for each organism. The inoculated plates were kept in the refrigerator for 1 hour to allow the extracts to diffuse into the agar (Atata, 2003). The Mueller Hinton Agar plates were incubated at 37°C for 24 hours. Antimicrobial activity was determined by measuring the diameter of zones of inhibition (mm) produced after incubation. 0.05% of Ciprofloxacin was used as control (NCCLS, 2000).

Determination of Minimum Inhibitory Concentration (MIC)

To measure the MIC values, various concentrations of the stock, 512, 256, 128, 64, 32, 16, 8, 4mg/ml were assayed against the test bacteria. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth (Prescott *et al.*, 1999; Shahidi Bonjar, 2004).

Determination of Minimum Bactericidal Concentration (MBC)

Equal volume of the various concentration of each extract and Mueller Hinton broth (Oxoid, UK) were

mixed in micro-tubes to make up 0.5ml of solution. 0.5ml of McFarland standard of the organism suspension was added to each tube (Shahidi Bonjar, 2004). The tubes were incubated aerobically at 37°C for 24 h. Two control tubes were maintained for each test batch. These include tube-containing extract without inoculum and the tube containing the growth medium and inoculum. The MBC was determined by sub culturing the test dilution on Mueller Hinton Agar and further incubated for 24 h. The highest dilution that yielded no single bacterial colony was taken as the Minimum bactericidal Concentration (Akinyemi et al., 2005). This was carried out on some of the extracts with high antimicrobial activity and some of the highly sensitive organisms

Results

Antimicrobial activities of the extracts obtained from *Bryophyllum pinnatum* and *Kalanchoe crenata* leaves, using different solvents and extracts from the squeezed leaves, against the tested organisms are shown in Table 1-3. The “omi ekan-ogi” extract of *Kalanchoe crenata* showed no activity. The zones of inhibition of each organism and the respective minimum inhibitory concentrations of the extracts, which range between 8mg/ml to 512mg/ml, are shown in Tables 1 and 2. The minimum bactericidal concentrations of some of the most active extracts, which range between 128mg/ml to 512mg/ml, are also shown in Table 3. Extracts from the squeezed leaves of *Kalanchoe crenata* was particularly active against tested organisms except *Candida albicans*. This was followed by the activities of extract from the local gin, aqueous extract and methanol extract. The methanol extract of *Bryophyllum pinnatum* showed greater antimicrobial activities than the local gin extract, followed by the squeezed leaves extract, then the aqueous extract, “Omidun” extract and palmwine extract. These extracts were active against tested organisms but the activity of the Palm-wine extract was poor.

Discussion

The result of this study showed that *Bryophyllum pinnatum* and *Kalanchoe Crenata* extracts have varied antibacterial activities against the tested organisms except “Omidun” extract of *Kalanchoe crenata* which showed no activity (Table 2). This suggests that the extracts of these plants are broad spectrum in their activities. This correlates with the observation of previous workers that plants contain substances that are antimicrobial (Olukoya, 1986). Extracts from the squeezed leaves of *Kalanchoe crenata* is the most active. It showed better antimicrobial activity than *Bryophyllum pinnatum* leaves that were prepared in the same way. It was active against both the Gram-positive and Gram-negative organisms with better activity against the Gram negative organisms. The palm wine extract of *Kalanchoe crenata* was highly active against the *E. coli* organisms tested, *Salmonella paratyphi* and *Citrobacter* spp. This implies that the use of palm-wine as a solvent releases some active ingredients which have high activity against some enteric organisms. There was no activity observed against *Candida albicans* (Table 2). The various antimicrobial activities of this plant extract in different solvents as shown from the result of this study, confirms its use traditionally in treating antimicrobial infections like dysentery, wound infection, sore, ear infection and abscesses. Both the aqueous extract of the dried leaves and methanol extraction of *kalanchoe crenata* showed moderate antimicrobial activities.

Out of all the extracts from *Bryophyllum pinnatum* leaves, the methanol extract was the most active. It showed marked antibacterial activities against Control strain of *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. The zones of inhibition observed in the Gram-positive organisms were almost the same with the control antibiotic (Ciprofloxacin) used (Table 1). This result is similar to the work of Akinpelu (2000) and Ofokansi (2005) that showed strong activities of methanol extract of *Bryophyllum pinnatum* against some Gram-positive organisms. The antimicrobial effect of methanol extract against these organisms may be due to the ability of the methanol to extract some of the active properties of these plants like phenolic compounds, saponin, bryophyllin and other secondary metabolites which are reported to be antimicrobial (Cowan, 1999; Okwu and Josiah, 2006). Extracts from the squeezed leaves of *Bryophyllum pinnatum* showed significant effect on some of the Gram positive and Gram-negative organisms (Table 2). This justifies the traditional use of the juice obtained from the slightly heated fresh leaves of this plant against antimicrobial infections like skin infections, wound infection, abscess and gastrointestinal disorder (Gill, 1992). This plant’s leaf juice is commonly used in treating ear infection and navel of newborn babies, which not only heals fast but also prevent infection at this site. This practice is common among the women in the Southern part of the country. Extracts of this plant in which “omidun” and the local gin were used as solvent were also found to have good activity on both Gram-positive and Gram-

Table 1: Antimicrobial Activities of Plants Extracts against Some Microorganisms

	ORGANISMS	ZONE OF INHIBITION (MM)												
		MB	SB	AB	PB	LB	OB	MK	SK	AK	PK	OK	LK	CIP
1	<i>Staphylococcus aureus</i> Control strain	20	17	16	11	22	17	14	21	16	-	-	20	21
2	<i>Staphylococcus aureus</i>	21	18	17	-	20	16	15	17	15	-	-	19	20
3	<i>Enterococcus faecalis</i>	18	23	18	-	20	24	11	20	16	-	-	18	14
4	<i>Bacillus subtilis</i>	23	24	19	15	21	24	18	30	19	-	-	17	24
5	<i>Escherichia coli</i>	16	18	-	-	19	16	12	22	14	30	-	11	31
6	<i>Escherichia coli</i> ATCC 25922	14	19	-	-	14	16	12	18	11	29	-	12	31
7	<i>Pseudomonas aeruginosa</i>	17	20	15	14	15	15	14	22	13	12	-	-	27
8	<i>Klebsiella pneumoniae</i>	12	16	-	-	12	18	12	23	10	-	-	-	32
9	<i>Shigella flexneri</i>	18	25	17	-	22	20	17	22	16	-	-	20	34
10	<i>Salmonella paratyphi</i>	17	18	-	-	18	17	10	18	14	30	-	-	31
11	<i>Citrobacter spp</i>	14	20	-	-	14	18	11	20	12	30	-	-	30
12	<i>Candida albicans</i>	17	-	-	-	16	-	23	-	-	-	-	17	25

KEYS

MB = Methanol extract of *Bryophyllum pinnatum* leaves OB = "Omi ekan ogi extract of *Bryophyllum pinnatum* leaves

SB = Extract from Squeezed leaves of *Bryophyllum pinnatum* slightly passed over heat AB = Aqueous extract of *Bryophyllum pinnatum* leaves SK = Extract from Squeezed leaves of *Kalanchoe crenata* slightly passed over heat. PB = Palmwine extract of *Bryophyllum pinnatum* leaves LB = Local gin extract of *Bryophyllum pinnatum* leaves MK = Methanol extract of *Kalanchoe crenata* leaves AK = Aqueous extract of *Kalanchoe crenata* leaves PK = Palmwine extract of *Kalanchoe crenata* leaves LK = Local gin extract of *Kalanchoe crenata* leaves OK = "Omi ekan ogi" extract of *Kalanchoe crenata* leaves
- = No zone of inhibition CIP = Ciprofloxacin

negative isolates tested. Other extracts of *Bryophyllum pinnatum* showed moderate to weak activity against tested organisms (Tables 1 and 2). This may be as a result of loss of some of the plant's active principle when drying or the inability of the solvents to dissolve some of the active principles of this plant (Ellof, 1998).

In this study, the various extracts from the two plants demonstrated varying activity but generally the traditional method of preparation of squeezing the leaves showed significant activity than other local and conventional methods of preparation. An exception was the methanol extract of *Bryophyllum pinnatum*. This study does not only show the scientific basis for some of the therapeutic uses of this plant in traditional medicine, but also confirms the fact that ethno botanical approach should be considered when investigating antimicrobial properties of plants (Adesanya et al., 2005; Iwu, 1993). It also explains why these plants give similar therapeutic result when they are used interchangeably in spite of their uniqueness (Sofowora, 1993).

The implication of the broad spectrum action of some of these extracts is that they can be useful in antiseptic and disinfectant formulation as well as in chemotherapy if the active principle can be isolated (Olukoya et al., 1993). The anti-pseudomonal and anti-staphylococcal activities of some of the effective extracts of these plants can be further explored.

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Table 2: Minimum Inhibitory Concentrations (mg/ml) of the Extracts

	ORGANISMS	SB	AB	OB	PB	LB	MB	SK	MK	PK	OK	AK	LK
1	<i>Staphylococcus aureus</i> Control strain	128	128	256	512	128	32	128	256	-	-	256	128
2	<i>Staphylococcus aureus</i>	256	128	256	-	128	32	128	512	-	-	256	128
3	<i>Enterococcus faecalis</i>	512	512	256	-	128	32	128	512	-	-	256	256
4	<i>Bacillus subtilis</i>	128	128	256	512	128	32	8	256	-	-	256	128
5	<i>Escherichia Coli</i>	256	-	512	-	512	256	64	256	256	-	512	512
6	<i>Escherichia Coli</i> ATTC 25922	256	-	512	-	512	256	64	256	256	-	512	512
7	<i>Pseudomonas aeruginosa</i>	256	512	512	256	128	64	8	512	512	-	512	-
8	<i>Klebsiella pneumoniae</i>	256	-	512	-	512	512	8	512	-	-	512	-
9	<i>Shigella flexneri</i>	256	256	256	-	256	256	32	256	-	-	256	256
10	<i>Salmonella paratyphi</i>	256	-	256	-	512	256	128	512	128	-	512	-
11	<i>Citrobacter spp</i>	256	-	512	-	512	512	64	512	256	-	512	-
12	<i>Candida albicans</i>	-	-	-	-	256	128	-	256	-	-	-	256

KEYS

MB = Methanol extract of *Bryophyllum pinnatum* leaves SB = Extract from Squeezed leaves of *Bryophyllum pinnatum* slightly passed over heat AB = Aqueous extraction of *Bryophyllum pinnatum* leaves PB = Palmwine extraction of *Bryophyllum pinnatum* leaves LB = Local gin extract of *Bryophyllum pinnatum* leaves OB = "Omidun" extract of *Bryophyllum pinnatum* leaves

MK = Methanol extract of *Kalanchoe crenata* leaves AK = Aqueous extract of *Kalanchoe crenata* leaves SK = Extract from Squeezed leaves of *Kalanchoe crenata* slightly passed over heat. PK = Palmwine extract of *Kalanchoe crenata* leaves LK = Local gin extract of *Kalanchoe crenata* leaves OK = "Omi ekan ogi" extract of *Kalanchoe crenata* leaves

- = No inhibitory activity

Table 3: Minimum Bactericidal Concentration (mg/ml) Of Some Extracts on Susceptible Organisms

Organisms	MB	SB	LB	PK	LK	SK
<i>Staphylococcus aureus</i> control strain	128	256	256	0	256	256
<i>Salmonella paratyphi</i>	-	-	0	-	-	-
<i>Bacillus subtilis</i>	128	-	-	-	256	-
<i>Enterococcus faecalis</i>	256	-	256	-	512	-
<i>Citrobacter spp</i>	-	-	-	0	-	-
<i>Shigella flexneri</i>	-	256	512	-	512	256
<i>Pseudomonas aeruginosa</i>	512	512	-	-	-	256
<i>Escherichia coli</i> ATCC 25922	-	0	-	0	-	-
<i>Escherichia coli</i>	-	-	-	0	-	0

KEYS

0=No bactericidal activity - = Not tested for minimum bactericidal activity MB = Methanol extract of *Bryophyllum pinnatum* leaves SB = Extract from Squeezed leaves of *Bryophyllum pinnatum* LB = Local gin extract of *Bryophyllum pinnatum* leaves SK = Extract from Squeezed leaves of *kalanchoe crenata* PK = Palmwine extract of *kalanchoe crenata* leaves LK = Local gin extract of *kalanchoe crenata* leaves

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