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Isolation and antibacterial activity of triterpenes from *Euphorbia kamerunica* Pax

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ABSTRACT

Two known compounds, friedelin and epifriedelinol were isolated from *Euphorbia kamerunica* Pax. The compound's structures were established on the basis of spectral analysis. The antibacterial activities of these compounds and the ethyl acetate extract were established on *Pseudomonas aeruginosa*, *Salmonella typhii* and *Staphylococcus aureus.* The extract displayed higher inhibition activities on the bacteria than the isolated compounds. The zones of inhibition of the extracts were between 9.0 and 30.5 mm, with the highest extract activity on *Staphylococcus aureus*. The minimum inhibition concentration (MIC) values of the isolated triterpenes were between 7.5 - 10 µg/ml.

Keywords: *Euphorbia kamerunica*, friedelin, epifriedelinol, antibacterial activity, MIC.

INTRODUCTION

Medicinal plants contain physiologically active principles which over the years have been exploited in traditional medicines for the treatment of various ailments (Adebanjo et al., 1983). A significant importance of medicinal plants is derived from its antimicrobial properties and these antimicrobial properties are of immense contribution to human health (Sokmen et al., 1999; Kelmanson et al., 2000; Srinivasan et al., 2001). Medicinal herbs constitute an indispensable component of traditional medicine practiced worldwide due to the low cost, perceived safety, bio-degradability, easy access and ancestral experiences (Martin-Bettolo, 1980; Wu-Yuan et al., 1988; Chen et al., 1989).

Traditionally, plants belonging to the Euphorbiaceae family have been used to treat skin infections, ulcers, warts, cancers, tumors, and disease of viral origin (Garcia-Barriga, 1974; Vasquez, 1982; Bernal and Correa, 1990; Pineros et al., 1992). With more than 1600 species in the Euphorbiaceace family, *Euphorbia* genus is the most representative of the family (Ozenda, 1991). *Euphorbia kamerunica* Pax belongs to this family which

is made up of trees, shrubs and herbs of the rain forest of Guinea and xerophytic habitats. The *Euphorbia* is known to contain skin irritants and co- carcinogenic principles in the form of esters of the structurally related diterpenes ingenane and daphanane (Hecker, 1978; Evans and Taylor, 1983; Hecker et al., 1984). Earlier studies undertaken on this genus have revealed the presence of triterpenes (Lima et al., 2003), diterpenes (Shi et al., 2005), macrocyclic diterpenes (Redei, et al., 2003) and aromatic compounds (Oksuz et al., 2002).

In this report, isolation of two triterpenes from *Euphorbia kamerunica* Pax is reported. The antibacterial activities of the crude ethyl acetate extract of the plant were studied to verify the folkloric use of the plant. Also, the antibacterial activities of the isolated triterpenes were determined and the minimum inhibitory concentration (MIC) estimated to pin down the bioactive component of the plant extract.

MATERIALS AND METHODS

General experimental procedures

IR spectra were recorded on Bruker FTIR vector 22 spectrophotometer in KBr discs, in cm^{-1}. EIMS (ionization voltage 70ev) was measured on a varian MAT 311/A mass spectrometer and HR EIMS were taken by MS JEOL-MS route, JMS-600H. ^{1}H-NMR at 200MHz and ^{13}C-NMR at 75MHz spectra on a Bruker AMX400. The chemical shifts (δ) are given in ppm, TMS as internal standard and coupling constant, *J* in H_z. Column chromatography of the crude extracts were carried out on silica gel 70-230 mesh (Kieselgel 60, Merck) and TLC was conducted on pre-coated plates (silica gel G60, Merck), detection by UV_{254} and by iodine.

Plant material

Euphorbia kamerunica Pax was collected from the Botanical gardens of the University of Ibadan, Nigeria and identified by Dr Abiodun Ayodele of the Herbarium of the Department of Botany and Microbiology, University of Ibadan. A voucher specimen with number 22278 was deposited at the herbarium.

Extraction and isolation

Fresh whole plant of *Euphorbia kamerunica* (10 kg) was washed, cut into pieces and pounded into pulp using mortar and pestle. The pulp was extracted with hexane and ethyl acetate successively. The extracts were filtered and water traces removed appropriately, evaporated to dryness under reduced pressure.

To obtain Tc (Friedelin), 43 g of the crude hexane extract of *Euphorbia kamerunica* Pax was subjected to column chromatography packed on silica gel 70-230 mesh (Kieselgel 60, Merck). Fractions 26 - 30 eluted with 5% diethyl ether in hexane were combined based on TLC analysis which afforded Tc. The crystal was re-crystalized in methanol several times yielding 35 mg of friedelin. Combined spectroscopic techniques were used in the analysis of Tc. Spectral and physical properties with literature data were used for identification of Tc as friedelin (Pretto et al., 2004; Grande et al., 1992).

Friedelin (Tc), white flakes. (R_f = 0.38, hexane/chloroform 2:1). M.p. 242 ^{0}C; IR bands (KBr): υ_{max} cm^{-1} = 2931.2, 2864.3, 1709.1 (C=O), 1624.1, 1460, 1380.9, 1308, 1186.4, 1113.5, 1076.9, 1004, 931.1, 797.1 UV (MeOH), λ_{max} nm (logΣ): 210(0.786).
^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 2.36 (3H, *m*), 2.0(1H, m), 1.22-1.88 (21H, *m*), 1.18 (3H, *s*), 1.16 (3H, *s*), 1.0 (5H, *s*), 0.92 (3H, *s*), 0.9 (6h, *d*, *J*=6.872 Hz), 0.725 (2H, s).

^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 22.5 (C-1), 39.9 (C-2), 210.6 (C-3), 59.7 (C-4), 39.5 (C-5), 36.6 (C-6), 18.5 (C-7), 53.3 (C-8) , 38.5 (C-9), 58.4 (C-10), 32.9 (C-11), 32.6 (C-12), 42.4 (C-13) , 41.8 (C-14), 32.3 (C-15), 36.2 (C-16), 35.6 (C-17), 42.9 (C-18), 37.7 (C-19), 32.0 (C-20), 41.5 (C-21), 35.3 (C-22), 7.1 (C-23), 18.9 (C-24), 20.5 (C-25), 14.9 (C-26), 18.2 (C-27), 28.4 (C-28), 30.2 (C-29), 30.7 (C-30).

The molecular formula is $C_{30}H_{50}O$ with molecular weight of 426. The exact mass is 426.3916 and elemental analysis showed: C, 84.44%; H, 11.81%; O, 3.7%.

Tm (epifriedelinol) was obtained from ethyl acetate extract (46 g) packed on column on silica gel 70-230 mesh (Kieselgel 60, Merck). Extraction with 5 % ether in hexane (fraction 24-26) yielded 28 mg of epifriedelinol which was purified in methanol. Combined spectroscopic techniques were used in the analysis of Tc and identification was done by comparison of spectral physical data with those of literature (Shirota et al., 1997; Grande et al., 1992).

Epifriedelinol (Tm), white powder with melting point of 279 °C. (R_f 0.30, hexane/chloroform, 2:1). IR bands (KBr): υ_{max} cm^{-1} = 3852, 3421, 2359, 1733, 1636, 1558, 1506, 1417, 1384, 1103, 1032, 536, 471. UV λ_{max} nm (MeOH) (log Σ): 230 (2.736).

^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 3.74 (1H, s), 1.1-2.1 (34H, m), 0.86-1.0 (28H, m).

^{13}C NMR (75 MHz, $CDCl_3$): 25.2 (C-1), 28.4 (C-2), 69.2 (C-3), 39.7 (C-4), 40.6 (C-5), 26.7 (C-6), 27.3 (C-7), 37.4 (C-8), 46.0 (C-9), 39.6 (C-10), 18.9 (C-11), 30.1 (C-12), 37.1 (C-13),49.6 (C-14), 23.9 (C-15), 37.1 (C-16), 30.3 (C-17), 47.9 (C-18), 29.6 (C-19), 27.8 (C-20), 29.6 (C-21), 33.3 (C-22), 9.5 (C-23), 17.8 (C-24), 18.4 (C-25), 15.6 (C-26), 18.7 (C-27), 18.7 (C-28), 22.8 (C-29), 20.1 (C-30).

Microorganisms and culture methods

Microorganisms used: *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella typhii* were clinical isolates from the Department of Medical Microbiology and Parasitology, University College Hospital (UCH), Ibadan, Nigeria. They were maintained on nutrient agar slants.

Quantitative antibacterial evaluation

The susceptibility tests were performed using the agar diffusion assay (Perez et al., 1990; Kavanagh 1972). Different concentrations of the isolated compounds (5.0 to 100 µg/ml) and crude ethyl acetate extract, (10 - 100 mg/ml) made in dimethyl sulfoxide (DMSO) were used for the assay. Gentamycin (10 µg/ml) was used as the standard antibiotic, while DMSO served as control. 0.1 ml of the bacteria (10^6 CFU/ml) was introduced into 15 ml of Mueller Hinton agar medium (Lab M) (NCCLS, 1993). These were distributed into Petri dishes which were kept at 4 ^{o}C for 2 h. A sterile cork borer 6.0 and 9.0 mm in diameter were used to cut wells on the agar plates for the isolated compounds and ethyl acetate extract respectively. After 24 h of incubation at 37 °C, the plates were observed for zones of inhibition. The minimum inhibitory concentration (MIC) values of the isolated compounds were taken as the lowest concentration of isolated compound that inhibited the growth of the bacteria after 24 h of incubation at 37 °C.

Statistical analysis

Results were expressed as mean ± S. E of two separate experiments. Statistical significance was determined using SPSS 10 software after one-way analysis of variance.

RESULTS

The antimicrobial activity of the ethyl acetate extract of *Euphorbia kamerunica* is shown in Table 1. The highest inhibitory activity on the microorganisms was recorded at 100 mg/ml and as the concentration of the extract decreased, the zone of inhibition also decreased. The extract has the highest impact on *S. aureus* with inhibition zone of 30.5±2.5 mm at 100 mg/ml. Gentamycin applied at 10 µg/ml and extract at 100 mg/ml had a comparative effects on *Pseudomonas aeruginosa* and *Salmonella typhi* but the extract exhibited a more pronounced effect on *Staphylococcus aureus*. At 10 mg/ml the extract had no activity on the bacteria. It is pertinent to note that the extract at all the concentrations was more active on *Staphylococcus aureus* than the standard antibiotic, gentamycin, though the extracts were applied at high concentrations. At high concentration the extracts displayed greater inhibitory activities on *S. Aureus* but at the lowest concentration (5 µg/ml) activity was completely lost. There was significant difference ($p<0.05$) between the extract inhibition ability on the microorganisms at different concentration levels.

Table 2 shows the antimicrobial activity of friedelin and epifriedelinol isolated from *Euphorbia kamerunica* on *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhi*.

In this study, friedelin (Tc) had its highest zone of inhibition, 15.50 mm on *S. aureus* at 100 µg/ml, 15.00 and 13.00 mm on *P. aeruginosa* and *S. typhi* respectively. At low concentration, 10 µg/ml, epifriedelinol activity could be described as medium, considering the zones of inhibition of 13.00, 12.50 and 10.00 mm on *P. aeruginosa*, *S. typhi* and *S. aureus* respectively, while that of friedelin 9.50, 10.00 and 10.00 mm respectively was low.

The Mininmum Inhibitory Concentration (MIC) of friedelin and epifriedilinol isolated from *Euphorbia kamerunica* is also shown in Table 2. The MIC of friedelin on *Pseudomonas aeruginosa* was 10 µg/ml, while on *Salmonella typhi* and *Staphylococcus aureus* it was 7.5 µg/ml. The MIC of epifriedelinol on *Pseudomonas aeruginosa* and *Staphylococcus aureus* was 7.5 µg/ml while that of *Salmonella typhi* was 10 µg/ml. For gentamycin the standard antibiotic used, the zones of inhibition of 23.00 mm, 21.00 mm and 14.00 mm was observed for *Pseudomonas aeruginosa, S. typhi* and *Staphylococcus aureus* respectively. DMSO, the negative control did not inhibit the growth of the microorganisms.

Table 1: Antimicrobial activity of the crude ethyl acetate extract of *Euphorbia kamerunica* using agar well diffusion technique.

	Zone of Inhibition (mm) ±S.D with different Concentration (mg/ml)						
Organism	**100**	**80**	**50**	**30**	**10**	**E. acetate**	**Gent**
P. aeruginosa	21.5±0.5a	19.5±2.5ab	19.0±1.0ab	13.5±2.5bc	9.0±0.0c	9.0±0.0c	23.0±0.0a
S. typhii	24.0±0.0a	22.0±1.0b	17.5±0.5c	11.0±1.0d	9.0±0.0c	9.0±0.0c	21.0±0.0b
S. aureus	30.5±2.5a	22.0±2.0b	19.0±1.0bc	14.0±2.0cd	9.0±0.0d	9.0±0.0c	14.0±0.0c

Means of two readings ± standard deviation; Values in the same row followed by the same letter are not significantly different ($p>0.05$) from each other.

GENT = Gentamycin, *P. aeruginosa = Pseudomonas aeruginosa, S. typhii = Salmonella typhii, S. aureus = Staphylococcus aureus.* Diameter of cork borer = 9.0 mm,

Table 2: Minimum Inhibitory Concentration (MIC) of compounds isolated from *Euphorbia kamerunica* on some microorganisms.

		Organisms /zone of inhibition (mm)		
Compound/concentration (µg/ml)		*P. aeruginosa*	*S. typhi*	*S. aureus*
Friedelin, Tc	100	15.00	13.00	15.50
	50	11.00	12.00	12.00
	10	9.5	10.00	10.00
	7.50	Nil	8.50	8.00
	5.00	Nil	Nil	Nil
Epifriedelinol, Tm	100	17.00	15.00	14.50
	50	13.00	13.00	12.50
	10	13.00	12.50	10.00
	7.50	9.50	Nil	7.50
	5.00	Nil	Nil	Nil
Gentamycin	10	23.00	21.00	14.00
DMSO control		6.00	6.00	6.00

P. aeruginosa = Pseudomonas aeruginosa, S. typhi = Salmonella typhi, S. *aureus = Staphylococcus aureus.* Diameter of cork borer = 6.00 mm

DISCUSSION

The IR spectrum of friedelin, Tc showed carbonyl (C=O) signal at 1709 cm^{-1} which is characteristic of ketone of friedelin derivatives (Budzikiewicz et al., 1963; Nozaki et al., 1986). Absorption bands at 2931 and 2864 cm^{-1} are due to C-H stretching vibration of the methyl groups, while bands at 1460 and 1070 cm^{-1} are due to C-H methyl bending vibrations from the cyclohexane rings. The IR signals agree with previous IR signals for friedelin isolated from various plants extracts (Tanaka and Matsunaga 1988). Friedelin and epifriedelinol co-exist as were earlier isolated from *Calophyllum brasiliense* and *C. inophyllum*. This study supports this finding as the two compounds were also isolated from *E. kamerunica*. These compounds are analgesic, antiviral, antiulcerogenic, anticancer, antibacterial, antitumor, antiviral and cytotoxic (Linuma et al., 1994; Satori et al., 1999; Yimdjo et al., 2004).

The UV signal at 210 nm is characteristic of triterpenes. The ^{1}H NMR signals between 0.8 - 1.4 ppm is mainly due to the proton of the angular methyl groups while those between 1.4 - 2.4 ppm are due to CH and CH_2 protons of the rings. All these chemical shifts are characteristic of the triterpenes (friedelin) and tally well with those of literature (Pretto et al., 2004; Grande et al., 1992). ^{13}C NMR spectrum of friedelin showed a chemical shift at 210.6 ppm due to the carbonyl carbon at position 3 (C-3). The carbons of the angular methyl groups are responsible for the resonance bands between 7.1 and 30.7 ppm while the quartenary carbon atoms are found in the following positions, C-3 (210.6 ppm), C-5 (39.5 ppm), C-9 (38.5 ppm), C-13 (42.4 ppm), C-14 (41.8 ppm), C-17 (35.6 ppm) and C-20 (32.0 ppm) while the remaining signals in the ^{13}C-NMR spectrum are the methine, CH and methylene CH_2 shifts. ^{13}C-NMR showed that the compound friedelin is made up of 30 carbon atoms while

DEPT analysis showed that there are four methine carbons, eleven methylene, eight methyl and seven quartenary carbons which confirmed its tritepenic nature. The (CH) signals are 59.7, 58.4, 53.3, 42.9, (CH_2): 41.8, 41.5, 39.5, 35.6, 35.8, 36.3, 32.9, 32.6, 30.7, 22.5, 18.4; methyl carbon (CH_3): 35.2, 32.3, 32.0, 20.5, 18.9, 18.17, 14.8, and 7.1 and quartenary carbons (C): 210.6, 49.2, 39.4, 38.5, 37.6, 30.0, 23.5. 2D ^1H-^1H COSY and other spectroscopic data help to elucidate the compound as friedelin with molecular formula $C_{30}H_{50}O$ and elemental analysis of C, 84.44%; H, 11.81% and O, 3.75% which was confirmed by literature data.

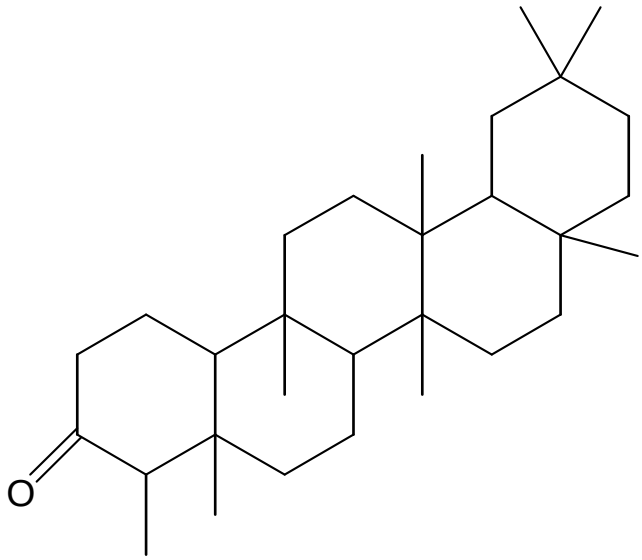
Epifridelinol, Tm is white powder with melting point of 279^{o}C. The UV shows an intense absorption at 230 nm (2.736) which is due to fused cyclic hexane rings. The ^1H-NMR spectrum showed a carbinol proton shift at 3.74 ppm. This hydroxyl is confirmed by IR absorption at 3421 cm^{-1}. The bands at 1636 and 1558 cm^{-1} are due to C-H vibration stretching. The ^1H-NMR signals between 1.00 and 0.86 ppm are those protons of angular methyl groups while CH_2 and CH protons accounted for remaining shifts between 2.0 and 1.2 ppm.^{13}C-NMR spectrum revealed that the compound is also a 30-carbon compound but with six quaternary carbons, eight methyl, eleven methylene, five methine groups from DEPT/APT analysis.

APT and DEPT indicated the signals of the carbon atoms thus; the eight methyl carbon - 35.1, 32.5, 32.0, 20.4, 18.9, 18.5, 16.6, and 11.9 ppm. Eleven methylene carbons - 41.9, 39.5, 36.3, 35.8, 35.5, 35.4, 33.0, 32.3, 30.9, 17.8, and 16.0 ppm. There are five methine carbons - 72.9, 61.5, 53.4, 49.4 and 42.9 ppm and six quartenany carbons – 49.3, 49.1, 48.7, 48.2, 38.5 and 28.3 ppm. Presence of signal 69.2 ppm confirmed carbinol carbon at position 3 (C-3). The UV signal recorded for the isolated epifriedelinol (Tm) at 230 nm is a characteristic of triterpenes of epifriedelinol type.

^{13}C NMR spectrum further showed a distinct C-OH shift at 61.52 ppm. Earlier, Ng et al. (2003), Shirota et al. (1997) and Betancor et al. (1980) had isolated this compound from *Aster tataricus* and *Eupatorium riparium* respectively, and their spectral data compared well with the spectral of the isolated epifriedelinol. To the best of our knowledge this is the first time these two compounds friedelin and epifriedelinol are been reported isolated from *Euphorbia kamerunica* Pax.

Medicinal plants are used in traditional medicine for several purposes. The secondary metabolites produced by plants constitute a source of bioactive substances and today scientific interest has increased due to search for new drugs of plant origin (Basile et al., 2000; Paiva et al., 2003).

A majority of the chemically useful antibiotics were active against the test strains at a level of at least 10 µg/ml. A pure agent that is not active at 100 µg/ml is unlikely to be a serious potential for clinical use unless it is active against a recalcitrant organism or is completely non-toxic (Mitscher et al., 1972). On the basis of this, tests on the activities of the isolates were carried out between 100 and 10 µg/ml. Epifreidelinol displayed the highest inhibition of 17.00 mm on *Pseudomonas aeruginosa* at 100 µg/ml. It also inhibited the growth of *Salmonella typhi* with inhibition zone of 15.00 mm and *Staphylococcus aureus* 14.50 mm at that same concentration. Epifriedelinol has been shown to have higher antimicrobial activity on microorganisms than friedelin (Pretto et al., 2004). The activity of the isolated compounds on the microorganism was low compared to crude extract though the concentration of application was not the same.



Friedelin (Tc)

This might be due to the fact that there are other active triterpenes or compounds in the extract that have not been isolated. Sainsbury (1970) reported that in plants, friedelin and epifriedelinol are often accompanied by other triterpenoids. *Euphorbia kamerunica* has both compounds present and other compounds as well; therefore synergism might be responsible for the higher crude extract activity on the microorganisms than the isolates (Odebode et al., 2004).

The stronger inhibition of crude extracts on *Staphylococcus aureus* observed is in accordance with the observation of other workers, and this is because the response of bacteria to antibacterial agents is influenced by their Gram staining properties (Sartori et al., 2003; Duffy and Power, 2001), which is attributed to the differences in their cellular composition. The cell wall of Gram-negative organisms is more complex than that of the Gram-positive bacteria.

Gram-negative bacteria cell wall consists of lipoprotein molecules covalently attached to the oligosaccharide backbone. In addition, on its outer side a layer of lipopolysaccharide (LPS) and protein attached by hydrophobic interactions and divalent metal cations. On the inner side is a layer of phospholipids, all of which may create a permeability barrier to lower concentrations of the drug which increase the inhibitory concentration within the cell (Hugo and Russel, 1983). This might be a reason why the extracts had greater activity on Gram-positive bacteria than the Gram-negative bacteria.

Conclusion

The folkloric usage of *E. kamerunica* is supported with the activities of both the isolates and the crude extract. Earlier phytochemical screening of the hexane, ethyl acetate and methanol extracts of the plant had revealed the presence of saponins, tannins, terpenoids, steroids, flavonoids and alkaloids (Sofowora, 1993) which are all physiologically bio-active on one microorganism or the other (Perrett et al., 1995; Peres et al., 1997).

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