



# ANTIMICROBIAL ACTIVITIES OF COMPOUNDS ISOLATED FROM *PYCNANTHUS ANGOLENSIS* (WELW.) WARB AND *BRYOPHYLLUM PINNATUM* (LAM) OKEN

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In the present study a semi-pure residue each from *Pycnanthus angolensis* (Welw.) Warb and *Bryophyllum pinnatum* (Lam) for the presence of biologically active compound(s) were investigated. This exercise led to two compounds whose identities have been established to be 1,6-dihydro-2-methyl-4-hydroxy-6-oxo-3-pyridine carboxylic acid ethyl ester (1,6-dihydro-2-methyl-4-hydroxy-6-oxo-3-nicotinic acid ethyl ester) (5-ethoxycarb, **NG5-a**) and 1-ethoxy-2-hydroxy-4-propenylguaethol (vanitrope, **KF-1a**), respectively using MS and IR spectral techniques. **NG-5a** was proved to be bacteriostatic against *Escherichia coli* but recorded no activity against *Staphylococcus aureus* and *Candida albicans*. **KF-1a** recorded only minimal activity against *S. aureus* but demonstrated no activities against *E. coli* or *C. albicans*.

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Nigeria) which served as a binding agent. The slurry obtained there from was vigorously shaken, thereby making it homogenous and free flowing. A thickness of 0.5 mm of the slurry was uniformly applied across the glass plates and allowed to set for 24 h. The coated plates were then activated in a laboratory oven (Gallenkamp, England) at 60 °C for at least 10 h prior to use.<sup>4</sup>

## Introduction

The seeds of *P. angolensis* are rich in palmitic, linoleic and linolenic acids as useful precursors in phytochemical biogenesis.<sup>1</sup> Furthermore, myristoleic acid (potent anti-arthritis agent) and tocotrienols (antioxidant and anti-inflammatory agents) have been obtained from the plant.<sup>2</sup> Also, four compounds namely, 3-ethoxy-3,7-dimethyl-1,6-octadiene (ethyl linalool), 1,2-benzenedicarboxylic acid diethyl ester (diethyl phthalate),<sup>3</sup> ethyl cinnamate and 9-oximino-2,7-diethoxy fluorene (2,7-diethoxy-9H-fluoren-9-one oxime)<sup>4</sup> have also been isolated from the ethyl acetate fraction of its leaves by column chromatography (CC) and preparative thin-layer chromatography (p-TLC) respectively.

The presence of cardiac glycosides, alkaloids, terpenes and tannins has also been indicated in *B. pinnatum*.<sup>5</sup> In addition, a steroid, 3-hydroxy-(3 $\beta$ ,17 $\beta$ )-spiro(andro-5-ene-17,1'-cyclobutan)-2'-one has been isolated from the butanol fraction of this plant by preparative thin-layer chromatography (p-TLC).<sup>3,6</sup> In continuation of work on these plants, residues coded **NG-5** and **KF-1** obtained from previous studies were subjected to preparative thin-layer chromatography (p-TLC) with the aim of isolating more compound(s) from the plants and as well as evaluating the antimicrobial activities of compound(s) so obtained.

## Experimental

### Preparation of plates

Similar 20 x 20 cm glass plates were washed in detergent solution, rinsed with water and air-dried. Silica gel (Sigma-Aldrich, USA) was treated with CaSO<sub>4</sub> (Bond Chemicals,

### Isolation of NG-5a

In order to isolate **NG-5**, the residue (deep brown, 65 mg) was painstakingly dissolved in some methanol and applied across the coated silica plate using a micro Pasteur pipette (Simax, India) 1 cm above the bottom edge of the plate and then allowed to dry. Afterward, the plate was developed in toluene:(CH<sub>3</sub>)<sub>2</sub>CO:H<sub>2</sub>O (40:80:4) inside a large chromatographic glass tank (Pyrex, USA). The obtained chromatogram showed two excellently resolved layers which were carefully scrapped, separately filtered with methanol and concentrated in vacuo on a rotary evaporator (R205D, shensung BS & T, China). The pure sub-fractions were monitored on commercial silica plates in toluene:(CH<sub>3</sub>)<sub>2</sub>CO:H<sub>2</sub>O (10:20:1) and (CH<sub>3</sub>)<sub>2</sub>CO:EtOAc (35:65) using FeCl<sub>3</sub>/CH<sub>3</sub>OH, Dragendoff's and vanillin-H<sub>2</sub>SO<sub>4</sub> as spray reagents. Further TLC evaluations indicated a spot in **NG-5a** (yellow compound, *R<sub>f</sub>* (0.22), 21 mg). C<sub>9</sub>H<sub>11</sub>NO<sub>4</sub>, MS (ES): *m/z* 197 (M<sup>+</sup>, 43.17 %), 179 (M-H<sub>2</sub>O<sup>+</sup>, 4.39 %), 151 (M-OC<sub>2</sub>H<sub>5</sub>-H<sup>+</sup>, 78.71 %), 139 (M-OC<sub>2</sub>H<sub>5</sub>-N+3<sup>+</sup>, 9.21 %), 123 (M-OC<sub>2</sub>H<sub>5</sub>-CO-H<sup>+</sup>, 88.49 %), 110 (M-OC<sub>2</sub>H<sub>5</sub>-N-CO<sup>+</sup>, 8.69 %), 95 (M-OC<sub>2</sub>H<sub>5</sub>-CO-CH<sub>3</sub>-OH+3<sup>+</sup>, 51.26 %), 83 (M-OC<sub>2</sub>H<sub>5</sub>-CO-CH<sub>3</sub>-OH-9<sup>+</sup>, 45.78 %), 69 (M-OC<sub>2</sub>H<sub>5</sub>-CO-CH<sub>3</sub>-N-9<sup>+</sup>, 62.02 %) and 42 (M-OC<sub>2</sub>H<sub>5</sub>-COO-CH<sub>3</sub>-N-OH-20<sup>+</sup> 100.00 %). FTIR: 717, 863 (alkyl substitution), 1076 (-C-O-C), 1621 (-C=C), 1715 (-C=O), 1732 (-C=O), 3456 (-NH) and 3567(-Ar-OH) cm<sup>-1</sup>.

### Isolation of KF-1a

The **KF-1** residue (yellow, 47 mg) was dissolved in some methanol and applied across the coated silica plate using a micro Pasteur pipette (Simax, India) 1 cm above the bottom edge of the plate and then allowed to dry. Afterward, the plate was developed in toluene: (CH<sub>3</sub>)<sub>2</sub>CO:H<sub>2</sub>O (40:80:4)

inside a large chromatographic glass tank (Pyrex, USA). The obtained chromatogram showed three layers which were carefully scrapped, separately filtered with methanol and concentrated in vacuo on a rotary evaporator (R205D, Shensung BS & T, China).

The pure sub-fractions were monitored on commercial silica plates in toluene:(CH<sub>3</sub>)<sub>2</sub>CO:H<sub>2</sub>O (10:20:1) and (CH<sub>3</sub>)<sub>2</sub>CO:EtOAc (35:65) using FeCl<sub>3</sub>/CH<sub>3</sub>OH and vanillin-H<sub>2</sub>SO<sub>4</sub> as spray reagents. Further TLC evaluations indicated a spot in **KF-1a** (amorphous pale yellow solid, *R<sub>f</sub>*(0.61), 0.18 mg).

**KF-1a**: C<sub>11</sub>H<sub>14</sub>O<sub>2</sub>, MS (ES) *m/z* 178 (M)<sup>+</sup> (100.00 %), 161 (M-OH)<sup>+</sup> (5.16 %), 149 (M-C<sub>2</sub>H<sub>5</sub>)<sup>+</sup> (54.91 %), 131 (M-OC<sub>2</sub>H<sub>5</sub>-2H)<sup>+</sup> (38.87 %), 121 (M-OC<sub>2</sub>H<sub>5</sub>-OH+5)<sup>+</sup> (7.33 %), 103 (M-C<sub>6</sub>H<sub>5</sub>+2)<sup>+</sup> (30.29 %), 91 (M-OC<sub>2</sub>H<sub>5</sub>-CH<sub>3</sub>-OH-10)<sup>+</sup> (20.81 %), 77 (M-C<sub>6</sub>H<sub>5</sub>-OH -7)<sup>+</sup> (27.89 %), 66 (M-C<sub>6</sub>H<sub>5</sub>-OH-CH<sub>3</sub>-3)<sup>+</sup> (12.82 %) and 55 (M-C<sub>6</sub>H<sub>5</sub>-OH-CH<sub>3</sub>-C<sub>2</sub>H<sub>5</sub>)<sup>+</sup> (21.80 %). FTIR: 767, 823 (alkyl substitution), 1056 (-C-O-C), 1618 (Ar-C=C), 1642 (exocyclic -C=C) and 3312 (Ar-OH) cm<sup>-1</sup>.

### Structural elucidation

The mass spectra of the compounds were obtained on Kratos MS 80 (Germany) while the infra-red analyses were done on Shimadzu FTIR 8400S (Japan).

### Antimicrobial screening

The microorganisms used in this study were limited to three *viz*: *Staphylococcus aureus* (ATCC 21824) (Gram positive), *Escherichia coli* (ATCC 23523) (Gram negative) and *Candida albicans* (NCYC 106) (fungus) were clinically isolated from specimens of diarrheal stool, abscesses, necrotizing fasciitis, urine and wounds obtained from the Medical Laboratory, University of Uyo Health Centre, Uyo. The clinical isolates were collected in sterile bottles, identified and typed by conventional biochemical tests.<sup>7,8</sup> These clinical microbes were then refrigerated at -5 °C. prior to use.

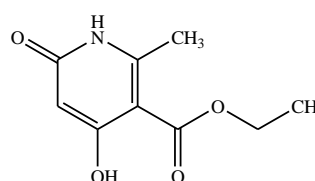
The media and plates were sterilized in an autoclave at 121 °C for 15 min. The hole-in-plate agar diffusion method was used observing standard procedure with Nutrient Agar-CM003, Mueller-Hinton-CM037 (Biotech Limited, Ipswich, England) and Sabouraud Dextrose Agar (Biomark, India) for the bacteria and fungus respectively. The inoculum of each micro-organism was introduced into each petri-dish (Pyrex, England). Cylindrical plugs were removed from the agar plates by means of a sterile cork borer (Simax, India) to produce wells with a diameter of approximately 6 mm. The wells were equidistant from each other and the edge of the plate (Washington, 1995, N.C.C.L.S, 2003).<sup>9,10</sup>

Concentrations of 20 mg mL<sup>-1</sup> of crude extracts of *P. angolensis* (CE<sub>p</sub>) and *B. pinnatum* (CE), 10 mg mL<sup>-1</sup> of ethyl acetate fraction (ET) and butanol fraction (BT), 2 mg mL<sup>-1</sup> of **NG-5a** and **KF-1a** were introduced into the wells. Also, different concentrations of 10 µg mL<sup>-1</sup> streptomycin (Orange Drugs, Nigeria), 1 mg mL<sup>-1</sup> of nystatin (Gemini Drugs, Nigeria) and deionized water were introduced into separate wells as positive and negative controls respectively.<sup>11</sup>

The experiments were carried out in triplicates. The plates were labeled on the underside and left at room temperature for 2 h to allow for diffusion. The plates were then incubated at 37± 2 °C for 24 to 48 h. Zones of inhibition were measured in mm with the aid of a ruler.

## Results and discussion

3-Pyridine carboxylic acid (3-nicotinic acid) and its derivatives are well-known compounds and can easily be identified by their MS and IR spectra. **NG-5a** was isolated and identified as an ethyl ester derivative of 3-nicotinic acid.



**Figure 1.** 1,6-Dihydro-2-methyl-4-hydroxy-6-oxo-3-pyridine carboxylic acid, ethyl ester (1,6-dihydro-2-methyl-4-hydroxy-6-oxo-3-nicotinic acid, ethyl ester, **NG-5a**).

It is pertinent to note that this could be due to partial esterification of 3-pyridine carboxylic acid in ethanol during extraction. In addition, **NG-5a** tested positive for the ferric chloride and Dragendoff's reagents indicating the presence of a hydroxyl group and an alkaloidal nucleus respectively.

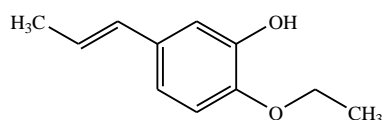
Due to the nature of its matrix, many fragmented ions could be seen in the mass spectrum of the compound. Those that are easily identifiable include (M)<sup>+</sup> at *m/z* 197 (43.17 %) while the peak at 179 (4.39 %) indicates the loss of water from the matrix. However, the fragments at 151(79.71 %) and 139 (9.21 %) represent the removal of ethoxy and ethoxy and nitrogen units respectively from the molecule. Furthermore, ions at 123 (88.49 %), 110 (28.69 %), 95(51.26 %)and 83 (45.78 %) correspond to the excisions of ethoxy and carbonyl, ethoxy, carbonyl and nitrogen and ethoxy, carbonyl, methyl and hydroxy groups respectively from **NG-5a**. The most abundant ion (base peak) at 42 (100 %) shows the removal of ethoxy, carboxylate, methyl, nitrogen and hydroxyl units from the molecular matrix.

The IR spectrum of the compound shows characteristic stretching bands at 717, 863, 1070, 1621, 1715, 1732, 3450 and 3567 cm<sup>-1</sup> indicating alkyl substitutions, ether linkage, endocyclic -C=C, carbonyl, -NH and aromatic hydroxyl absorptions respectively.

The chemical structure of **KF-1a** was established by a combination of spectroscopic techniques as highlighted above. These data were matched with those in the library data of organic compounds and were found to be consistent with those in literature. Consequently, **KF-1a** (Figure 2) has been identified to be 1-ethoxy-2-hydroxy-4-propenyl guaethol (vanitrope).

**Table 1.** Antimicrobial screening of crude extract, ethyl acetate fraction, **NG-5a** and **KF-1a** at different concentrations on test microbes in water.

Test microbe	CE <sub>p</sub> /CE 20 mg mL <sup>-1</sup>	ET/BT 10 mg mL <sup>-1</sup>	NG-5a 2 mg mL <sup>-1</sup>	KF-1a 2 mg mL <sup>-1</sup>	deionized water	SP 10 µg mL <sup>-1</sup>	NY 1mg mL <sup>-1</sup>
<i>S. aureus</i>	6	6	6.5	7.5	6	27	6
<i>E. coli</i>	6	6	11.5	6	6	28	6
<i>C. albicans</i>	6	6	6	6	6	6	25

**Figure 2.** 1-Ethoxy-2-hydroxy-4-propenyl guaiacol (**KF-1a**).

Due to the nature of the matrix, many fragmented peaks appeared in the MS of the compound. Those that are easily identifiable include (M)<sup>+</sup> which shows as the most abundant ion (base peak) at *m/z* 178 (100.00 %) while fragments at 161 (5.16 %) and 149 (54.41 %) represent the loss of hydroxy and ethyl groups from the molecule, respectively. Furthermore, the ions at 103 (30.29 %), 77 (27.89 %) 66 (12.82 %) and 55 (21.80 %) indicate the disintegration of the molecular matrix by the excisions of phenyl and some smaller units such as methyl and hydroxy from (M)<sup>+</sup>. However, peaks at 131 (38.87 %) and 121 (7.33%) reveal the removal of ethoxy groups from the compound.

The IR spectrum of **KF-1a** shows absorptions at 767, 823, 1056, 1618, 1642 and 3312 cm<sup>-1</sup> indicating diagnostic alkyl substitutions, an ether linkage (-C-O-C), aromatic -C=C, exocyclic -C=C and aromatic -OH functional groups respectively. It should be noted that **KF-1a** was isolated with a sweet fragrance characteristic of oils, spices, perfumes and food additives.<sup>16</sup>

#### Antimicrobial tests

The spectrum of microbes employed in these tests was narrow, encompassing one each of gram-positive (*S. aureus*) and gram negative (*E. coli*) bacterial strains and a fungus (*C. albicans*). The results presented in Table 1 show that the crude extracts and fractions were inactive against *S. aureus*, *E. coli* and *C. albicans*. However, **NG-5a** was appreciably bacteriostatic against *E. coli*. It but recorded no activities against *S. aureus* and *C. albicans*., **KF-1a** was minimally active against *S. aureus* but recorded no activities against *E. coli* or *C. albicans*. The activity given by **NG-5a** against *E. coli* has importance because of the resistance of this microorganism against known antimicrobial agents. This resistance is believed due to the presence of a three-layered envelope which does not allow permeation of external agents. The compounds demonstrated no antifungal activity against *C. albicans*.

#### Conclusion

The isolation of these compounds is being reported for the first time from the plants. Hence, **NG-5a** and **KF-1a** are expected to serve as chemotaxonomic markers for both plants. Furthermore, the results of the antimicrobial screening lend some justification to the uses of these plants especially in the treatment /management of some bacterial infections. However, the compounds will be screened against other bacterial and fungal strains in the future with the aim of obtaining better activities.

#### Conflict of Interest

The authors declare no conflict of interest

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