ETHYL LINALOOL AND DIETHYL PHTHALATE FROM PYCNANTHUS ANGOLENSIS (WELW.) WARB.

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Pycnanthus angolensis (Welw.) Warb. (Family; Myristiceae) leaves were extracted cold with 50 % ethanol and the obtained aqueous crude extract partitioned with ethyl acetate. Furthermore, the ethyl acetate fraction was subjected to silica gel column chromatography and the isolated chemical compounds tested for antimicrobial activity against *Staphylococcus aureus, Escherichia coli* and *Candida albicans*. The identities of two isolates have been revealed to be 3-ethoxy-3,7-dimethyl-1, 6-octadiene (ethyl linalool) and diethyl phthalate (1,2-benzenedicarboxylic acid diethyl ester) using the MS and IR spectral techniques. Both compounds showed strong bacteriostatic action against *E. coli* but were inactive against *S. aureus* and *C. albicans*.

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INTRODUCTION

Pycnanthus angolensis (Welw.) Warb**.** syn. *P. kombo* (known as African or wild or false nutmeg) was originally native to the forest zones of West and Central Africa but now cultivated in and around the world.[1] Different preparations of the plant are employed in diverse African folklores to treat chest infections, diabetes, lumbago, wounds, arthritis, anaemia, mouth-thrush, malaria, leprosy, toothache, infertility, sexually transmitted disease and skin-fungal infections.[2-4] The larvicidal and antitumor potentials of the plant have been studied [5-6] while reports of isolation of flavonoids and terpenes from the bark and roots and quinones from the leaves abound.[7] This present investigation aimed at isolating the compound(s) from the ethyl acetate fraction which demonstrated the highest antimicrobial activity in a previous bioactivity-guided fractionation study of the plant.[8] In addition, the compounds so obtained will be screened for antimicrobial activity with the aim of confirming or disproving the claims highlighted in traditional medicine especially for the treatment/management of bacterial infections.

MATERIALS AND METHODS

The fresh leaves of *P. angolensis* were collected around April, 2016 within the precinct of University of Uyo, Akwa Ibom State, Nigeria. The plant had previously been identified in a study. [8] Immediately after collection, the plant was dried in a laboratory oven (Gallenkamp, England) at 40 ^{0}C for 48 h and the resultant material powdered on an electric mill (Uniscope, England).

Extraction and isolation

The dried powder (1.1 kg) was exhaustively extracted with 50 % EtOH (3 x 5L) at room temperature (27± 2 ^{0}C) for 72 h. The resultant crude extract mixture was filtered, concentrated *in vacuo* on a rotary evaporator. 250 g of dried crude extract was obtained and then stored in a desiccator prior to further use. Consequently, 15 g of the extract was partitioned using H_2O: EtOAc (8 x 200 mL). The combined ethyl acetate fractions were evaporated to dryness to give a brown solid residue. Hence, 1.2 g of the fraction was chromatographed on a silica gel 254 column (Pyrex, USA; 10 g pre-swollen in 100 % toluene; 2 g concentration zone + 8 g separation zone; 13.6 x 4 cm) and eluted with a gradient of 20 % $(CH_3)_2CO$: toluene (48 mL), 30 % $(CH_3)_2CO$: toluene (48 mL), 40 % $(CH_3)_2CO$: toluene (48 mL), 50 % $(CH_3)_2CO$: toluene (48 mL), 60 % $(CH_3)_2CO$: toluene (48 mL) 70 % $(CH_3)_2CO$: toluene (48 mL) and 80 % $(CH_3)_2$ CO: toluene (48 mL). Fractions of 8 mL each were collected and monitored on silica plates in $(CH_3)_2CO$: toluene: H_2O (10:20:1) using $FeCl_3/CH_3OH$ and vanillin-H_2SO_4 as spray reagents.

Hence, fractions with similar TLC characteristics (R_f values, reaction with vanillin-H_2SO_4 spray) were bulked and dried. Five sub-fractions coded NG-1, NG-2, NG-3, NG-4 and NG-5 were obtained. Further TLC examinations of these sub-fractions in $(CH_3)_2CO$: toluene: H_2O (10: 20: 1) and $(CH_3)_2CO$: EtOAc (35: 65) indicated a single spot in **NG-2** (pale yellow compound; R_f (0.53); 62 mg) while the others showed multi-component TLC profiles. Attempts were made to clean up the semi-pure residues separately NG-1, NG-3, NG-4 and NG-5 on a short silica gel 254 column (7.8 x 4 cm) using 50 % $(CH_3)_2CO$: toluene (48 mL). However, only NG-4 furnished a single spot. Hence, **NG-4c** was isolated (off-white compound; R_f (0.24); 36 mg). The refractive indices and optical rotation were obtained using WAY-15 Abbe refractometer (England) and ADP-220 Bellingham Stanley polarimeter (England) respectively. Refractive indices and optical rotation were measured at the wavelength (λ) of Na^D line (589.3 nm) and 20 °C.

Antimicrobial tests

The microorganisms used in this study, namely; *Staphylococcus aureus* (ATCC 21824), *Escherichia coli* (ATCC 2353) and *Candida albicans* (NCYC 106) 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 convectional biochemical tests.[9-10] These clinical microbes were then refrigerated at -5 ^{0}C. The agar plates used were prepared by adhering to the manufacturer's instructions. 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 procedures for Nutrient Agar-CM003, Mueller-Hinton-CM037 (Biotech Limited, Ipswich, England) and Sabouraud Dextrose Agar (Biomark, India) in respect of 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 using a sterile cork borer (Simax, India) to produce wells with a diameter of approximately 5 millimetres. The wells were equidistant from each other and the edge of the plate.[11-12] Concentrations of 20 mg mL^{-1} of crude extract, 10 mg mL^{-1} of ethyl acetate fraction, 2 mg mL^{-1} of **NG-2** and **NG-4c** were introduced into the wells. Also, different concentrations of 10 µg mL^{-1} Streptomycin (Orange Drugs, Nigeria), 1 mg mL^{-1} of nystatin (Gemini Drugs, Nigeria) and deionized water were introduced into separate wells as positive and negative controls respectively.[5,13-15] The experiments were carried out in triplicates. The plates were labelled on the underside and left at room temperature for 2 h to allow for diffusion. The plates were then incubated at 37 ± 2 ^{0}C for 24 to 48 h. Zones of inhibition were measured in millimetres (mm) with the aid of a ruler.

RESULTS AND DISCUSSION

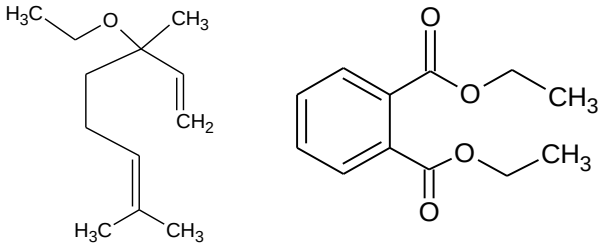
The two isolated compounds were identified as 3-ethoxy-3,7-dimethyl-1,6-octadiene (ethyl linalool) and diethyl phthalate (1,2-benzenedicarboxylic acid diethyl ester) respectively (Figure 1**)**. The refractive indices of **NG-2** and **NG-4c** were found to be 1.4009 and 1.5006 respectively. These values are consistent with the literature values (1.4006 and 1.5002 given for ethyl linalool and diethyl phthalate respectively.

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).

NG-2: $C_{12}H_{22}O$; pale yellow compound; R_f (0.53); $[\alpha]_D^{20}$ (+3º); $[n]_D^{20}$ (1.4009); MS [ES+-MS] m/z (relative intensity): 182 $[M]^+$ (0.64 %), 180 $[M-2H]^+$ (0.70 %), 166 $[M-CH_3-1H]^+$ (4.05 %), 150 $[M-2CH_3-2H]$+ (3.30 %), 137 $[M-3CH_3]$+ (3.20 %), 121 $[M-OC_2H_5-CH_3-1]$+ (41.01 %), 107 $[M-OC_2H_5-2CH_3]$+ (8.04 %), 96 $[M-OC_2H_5-3CH_3+4]$+ (51.30 %), 71 $[M-OC_2H_5-3CH_3-21]$+ (90.13 %), 69 $[M-OC_2H_5-3CH_3-23]$+ (30.07 %) and 43 $[M-M+OC_2H_5-2]$+(100.00 %); IR [FTIR] cm-1: 923, 912, 876 (alkyl substitution), 1652 (acyclic -C=C) and 1087 (-C-O).

NG-4c: $C_{12}H_{14}O_4$ (off-white compound; R_f (0.24); $[\alpha]_D^{20}$ (0º); $[n]_D^{20}$ (1.5006); MS [ES+-MS] m/z (relative intensity): 222 [M]+ (1.42 %), 194 $[M-C_2H_5+1]$+ (3.30 %), 177 $[M-OC_2H_5]$+ (37.73 %), 164 $[M-OC_2H_5-CH_3-2]$+ (4.06 %), 149 $[M-OC_2H_5-2CH_3+2]$+ (100 %) 132 $[M-2OC_2H_5]$+ (5.26 %), 121 $[M-2OC_2H_5-11]$+ (6.08 %), 105 $[M-2OC_2H_5-CO-1]$+ (7.31 %), 93 $[M-2OC_2H_5-CO-11]$+ (7.50 %), 78 $[M-2OC_2H_5-2CO+2]$+ (23.05 %), 65 $[M-2OC_2H_5-2CO-11]$+ (18.26 %) and 50 $[M-2OC_2H_5-2CO-25]$+ (20.15 %); IR [FTIR] cm-1: 932, (alkyl substitution), 1072 (-C-O-C), (1602) Ar (-C=C) and 1721 (-C=O).



NG-2 **NG-4c**

Figure 1. 3-ethoxy-3, 7-dimethyl-1, 6-octadiene (ethyl linalool, **NG-2**) and 1, 2-benzenedicarboxylic acid diethyl ester (diethyl phthalate, **NG-4c**).

In the mass spectrum of ethyl linalool, the molecular peak could be assigned easily at m/z 182 (0.64 %) while fragments at 166 (4.05 %), 150 (3.30 %) and 137 (3.20 %) represent the excision of methyl group(s) from $[M]^+$. Furthermore, ions at 121 (41.01%), 107 (8.04 %), 96 (51.30 %), 71 (90.13 %) and 69 (30.07 %) correspond to the losses of ethoxy and methyl groups from **NG-2.** The peak at 43 (100 %) (base peak) indicates the disintegration of the molecule save for an ethoxy group.

The FTIR spectrum of **NG-2** shows absorptions at 1652 and 1087 cm^{-1} indicating acyclic -C=C and -C-O-C (ether linkage) respectively. The compound, ethyl linalool showed an optical rotation of +3° indicating dextrorotation.

Equally, **NG-4c** (diethyl phthalate) showed $[M]^+$at m/z 222 (0.12 %), while ions at 177 (37.73 %), 132 (5.26 %) and 121 (6.08 %) indicate the loss of ethoxy group(s) from the molecule. In addition fragments at 164 (4.06 %) and 149 (100%) (base peak) correspond to the excisions of ethoxy and methyl group(s) from **NG-4c.** Furthermore, ions at 105 (7.31 %), 93 (7.50 %), 78 (23.05 %) 65 (18.26 %) and 50 (20.15 %) show the removal of ethoxy and carbonyl group(s) from $[M]^+$.

The IR spectrum of **NG-4c** shows diagnostic stretchings at 1721, 1602 and 1072 cm^{-1} representing (-C=O), Ar (-C=C) and-C-O-C (ether linkage) functional groups respectively.

Table 1. Results of antimicrobial screening of crude extract, ethyl acetate fraction, **NG-2** (ethyl linalool) and **NG-4c** (diethyl phthalate) at different concentrations on test microbes in deionized water

Species	CE, 20 mgmL^{-1}	ET, 10 mgmL^{-1}	NG-2, 2mg mL^{-1}	NG-4c, 2 mg mL^{-1}	H_2O	SP, 10 µg mL^{-1}	NY, 1mg mL^{-1}
S. aureus	5	5	5	5	5	26	5
E. coli	5	5	17	15	5	31	5
C. albicans	5	5	5	5	5	5	29

Key: The zone diameter recorded is zone of inhibition + size of cup (zone of inhibition +5) mm; **CE** = Crude ethanolic extract; ET = Ethyl acetate fraction; S**P** =Streptomycin; **NY** =Nystatin;**NG-2**=3-ethoxy-3,7-dimethyl-1,6-octadiene (ethyl linalool);**NG-4c =** Diethyl phthalate (1,2-benzenedicarboxylic acid diethyl ester);**NCTC** - National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9, UK.**NCYC**- National Collection of Yeast Cultures, UK. **ATCC**- American Type Culture Collection**,** Washington, DC. *S. aureus* (ATCC 21824), *E. coli* (ATCC 23523), *C. albicans* (NCYC 106).

Antimicrobial screening

The spectrum of microbes employed in the sensitivity 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 displayed in **Table** 1 show that the crude extract, ethyl acetate fraction, **NG-2**, and **NG-4c** were inactive against *S. aureus* and *C. albicans.* However, the two compounds were remarkably bacteriostatic against *E. coli.* This result was unexpected because gram-negative bacteria are well known for their unique resistance to antimicrobial agents. This resistance is believed to be due to the nature of the cell envelope of these organisms which unlike gram-positive organisms possess a sophisticated three-layered envelope which does not allow permeation of external agents. Also, both compounds demonstrated no antifungal activity against *C. albicans.* This particular observation was to be expected because of fungal strains especially *Candida spp*. limit the permeation of substances because of their integral structures which are pleomorphic and facultative in nature hence, resembling those of higher plants.[16] It is instructive to mention that derivatization studies are currently on-going in our laboratories with the aim of improving on the observed activity.

CONCLUSION

The isolation of the two compounds is being reported for the first time from the ethyl acetate fraction of the plant. Hence, ethyl linalool and diethyl phthalate are expected to serve as chemotaxonomic markers for this species and the genus, *Pycnanthus* in general. Furthermore, the results of the antimicrobial sensitivity tests lend some credence to the use of this plant especially in the treatment or management of the bacterial disease.

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