ISOLATION OF ETHYL CINNAMATE AND A SUBSTITUTED FLUORENE FROM *PYCNANTHUS ANGOLENSIS* (WELW.) WARB

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Before now, two compounds namely, 3-ethoxy-3,7-dimethyl-1,6-octadiene (ethyl linalool) and 1,2-benzenedicarboxylic acid diethyl ester (diethyl phthalate) have been isolated from the ethyl acetate fraction of *Pycnanthus angolensis* by column chromatography. In this study, the preparative thin-layer chromatography (p-TLC) of two previously obtained residues was carried out. The chemical and biological properties of the compounds obtained there from were evaluated. This exercise led to two isolates (**NG-1b** and **NG-3b)** whose identities have been revealed to be ethyl cinnamate **(**cinnamic acid, ethyl ester) and 9-oximino-2,7-diethoxy fluorene (2,7-diethoxy-9H-fluoren-9-one oxime) respectively using the MS and IR spectral techniques. The compounds were surprisingly strongly bacteriostatic against *Escherichia coli* but recorded no activity against *Staphylococcus aureus* and *Candida albicans.*

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Introduction

Pycnanthus angolensis (Welw.) Warb. is synonymous with *P. kombo, Myristica angolensis* (Welw.) and *Myristica kombo* (Baill.) amongst many others. Apart from its diverse uses in traditional medical practice, the plant is also employed in economic ventures such as fuel and paper pulp, candle, plywood, timber, furniture making and construction associated with paneling, siding, roof shingles and framing.[1] In a previous study two compounds namely, 3-ethoxy-3,7-dimethyl-1,6-octadiene (ethyl linalool) and 1,2-benzene-dicarboxylic acid diethyl ester (diethyl phthalate) from ethyl acetate fraction by column chromatography were obtained.[2] Preparative thin-layer chromatographic(p-TLC) study has been carried out on two of obtained residues which showed multi-component TLC profiles with the aim of isolating more compounds from the plant. The antimicrobial potential of obtained compounds was also evaluated.

Experimental

Preparation of plates

Glass plates (20 x 20 cm) were washed in detergent solution, rinsed with water and air-dried. Silica gel (Sigma-Aldrich, USA) was treated with $CaSO_4$ (Bond Chemicals, 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 ^{0}C for at least 10 h prior to use.

Isolation

NG-1 (light brown, 78 mg) and **NG**-3 (deep brown, 46 mg) were separately dissolved in a little methanol and applied across the coated silica plates using a micro Pasteur Pipette (Simax, India) 1 cm above the bottom edge of the plates and then allowed to dry. Afterwards, the plates were separately developed in toluene:acetone:water (40:80:4) inside large chromatographic glass tanks (Pyrex, USA). The obtained chromatograms showed appreciably distinct layers which were carefully scrapped, separately filtered with methanol and concentrated in vacuo in a rotary evaporator (R205D, Shensung BS & T, China). The apparently pure sub-fractions were monitored on commercial silica plates in toluene:acetone:water (10:20:1) and acetone:ethyl acetate (35:65) using $FeCl_3/CH_3OH$ and vanillin-H_2SO_4 as spray reagents. Further TLC evaluations indicated a spot in **NG-1b** (yellow oil; R_f (0.48); 37 mg) and **NG-3b** (pale brown compound; R_f (0.31), 18 mg).

Structural elucidation

The mass spectra of the compounds were obtained on Kratos MS 80 (Germany) while the IR analyses were done on Shimadzu FTIR 8400S (Japan). The refractive index was obtained using WAY-15 Abberefractometer (England). The refractometer was initially zeroed and the refractive index measured at the wavelength (λ) of Na-D line (589.3 nm) and 20 ^{0}C.

Antimicrobial screening

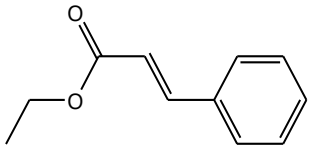
The micro-organisms used in this study were limited to three *viz*: one Gram (+), Gram (-) and a fungus. *Staphylococcus aureus* (ATCC 21824), *Escherichia coli* (ATCC 23523) and *Candida albicans* (NCYC 106) were

clinically isolated from specimens of diarrheal stool, abscesses, necrotizing fascitis, 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.[3, 4] These clinical microbes were then refrigerated at -5 ^{0}C at the Microbiology and Parasitology Unit, Faculty of Pharmacy 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 microorganism 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 5 mm. The wells were equidistant from each other and away from the edge of the plate.[5, 6] Concentrations of 20 mg mL-^{1} of crude extract, 10 mg mL-^{1} of ethyl acetate fraction, 2 mg mL^{-1}of **NG-1b** and **NG-3b** 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 was introduced into separate wells as positive and negative controls respectively.[7] 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 with the aid of a ruler.

Results and Discussion

Spectroscopic data

NG-1b: C_{11} H_{12} O_2, yellow oil, R_f = 0.48, $[n]_D^{20}$ = 1.5583. MS [ES$^+$-MS] *m/z* 177 $[M+H]^+$ (100.00 %), 176 $[M]^+$ (0.70 %), 161 $[M\text{-}CH_3]^+$ (11.29 %), 147 $[M\text{-}C_2H_5]^+$ (83.34 %), 131$[M\text{-}OC_2H_5]^+$(54.40 %), 122$[M\text{-}C_2H_2\text{-}CO]^+$ (5.61%), 99 $[M\text{-}C_6H_5]^+$ (75.94 %) and 54 $[M\text{-}C_6H_5\text{-}OC_2H_5]^+$. IR [FTIR] cm^{-1}: 785, 873 (alkyl substitution), 1072 (-C-O-C), 1616 (Ar-C=C), 1637 (exocyclic-C=C) and1712 (-C=O).

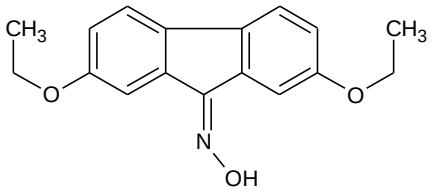


NG-1B

Figure 1. Ethyl cinnamate.

NG-3b:$C_{17}H_{17}NO_3$, pale brown compound, R_f = 0.31. MS [ES$^+$-MS] *m/z* 283$[M]^+$ (1.26 %), 266[M-OH]$^+$ (1.07 %), 254$[M\text{-}C_2H_5]^+$(24.70 %), 238$[M\text{-}OC_2H_5]^+$ (1.53 %), 226 $[M\text{-}2C_2H_5+1]^+$ (32.36 %), 210 $[M\text{-}OC_2H_5\text{-}C_2H_5+1]^+$ (5.23 %), 198 $[M\text{-}2OC_2H_5+5]^+$ (2.36%), 181 $[M\text{-}C_6H_5\text{-}O\text{-}9]^+$ (7.81 %), 163 $[M\text{-}2OC_2H_5\text{-}OH\text{-}N+1]^+$ (5.21 %), 149 $[M\text{-}2OC_2H_5\text{-}OH\text{-}N\text{-}13]^+$ (12.46 %), 129 $[M\text{-}C_6H_5\text{-}OC_2H_5\text{-}OH\text{-}N\text{-}1]^+$ (4.15 %), 115$[M\text{-}C_6H_5\text{-}OC_2H_5\text{-}CH_3\text{-}OH\text{-}N]^+$ (9. 36 %), 101 $[M\text{-}C_6H_5\text{-}OC_2H_5\text{-}C_2H_5\text{-}OH\text{-}N]^+$ (8.28 %), 94 $[M\text{-}C_6H_5\text{-}OC_2H_5\text{-}C_2H_5\text{-}OH\text{-}N\text{-}7]^+$ (21.45 %), 81 $[M\text{-}2C_6H_5\text{-}OC_2H_5\text{-}13]^+$ (27.15 %), 58$[M\text{-}2C_6H_5\text{-}OC_2H_5\text{-}OH\text{-}N\text{-}5]^+$ (100 %) and 55 $[M\text{-}2C_6H_5\text{-}OC_2H_5\text{-}OH\text{-}N\text{-}8]^+$ (32.75 %). IR [FTIR] cm^{-1} 756, 758, 856, 859 (alkyl substitution), 1047, 1048 (-C-O-C), 1606, 1609 Ar (-C=C), 2219 (-C=N) and 3471 (-CN-OH).

The determination of a physical constants such as the refractive index is used in the qualitative and quantitative analyses of substances. It is also employed to confirm the purity, identity, the integrity of compounds and as well as monitor the progress of reactions. **NG-1b** was isolated as an oily substance with a fruity fragrance. Consequently, the refractive index of **NG-1b** was determined at the wavelength (λ) of Na-D light (589.3 nm) and at 20 ^{0}C. The refractive index of a substance is an indication of the number, type of atoms and chemical groups (species) in the substance. Each atom or group in the substance contributes to its refractivity which adds eventually to the refractive index of the substance.**NG-1b** recorded a refractive index of 1.5583which is consistent with the literature value of 1.5880. However, the refractive index of **NG-3b** could not be unambiguously determined because of small sample size.



NG-3b

Figure 2. 2,7-Diethoxy-9*H*-fluoren-9-one oxime.

The identities of the compounds were established by a combination of spectroscopic techniques as shown above. The obtained data were compared with those in the library data of organic compounds. Furthermore, these data were found to be consistent with those reported in the literature.[8-11] Consequently, **NG-1b** and**NG-3b** have been revealed to be ethyl cinnamate **(**cinnamic acid, ethyl ester) and 9-oximino-2, 7-diethoxy fluorene (2, 7-diethoxy-9H-fluoren-9-one oxime) respectively as presented in 1igures1 and 2. Due to the nature of the matrices, many fragmented ions appeared in the mass spectra of the compounds. In the MS of **NG-1b**, those that are easily identifiable include $[M]^+$ at *m/z* 176 (0.70 %) while fragments at 161 (11.29 %) and 147 (83.34 %) represent the removal of methyl and ethyl units respectively from molecular ion. Furthermore, peaks at 131 (54.40 %) and 99 (75.94 %) correspond to the excisions of ethoxy and phenyl groups from **NG-1b** while the peak at 77 (100 %) (base peak) indicates the addition of a proton to the molecule. The FTIR spectrum of **NG-1b** shows diagnostic stretchings at 785, 873, 1072, 1616, 1637 and 1712 cm^{-1} indicating alkyl substitutions, ether linkage, aromatic C=C, exocyclic -C=C and C=O absorptions respectively. The mass spectrum of **NG-3b** shows very elaborate fragmentations in its matrix. The molecular ion can readily be identified at *m/z* 283 (1.26 %) while the ion at 266 (1.07 %) indicates a loss of a hydroxyl group. The fragments at 254 and 226 correspond to the excision of ethyl units from the $[M]^+$. The peaks at 198 (2.36 %), 163 (5.21 %) and 149 (12.46 %) represent the successive losses of two ethoxy and an oximino group respectively from the matrix.

Table 1. Antimicrobial screening of crude extract, ethyl acetate fraction, **NG-1b** and *NG-3b* at different concentrations on test microbes in water.

Test microbe	CE 20 mg mL^{-1}	ET 10 mg mL^{-1}	NG-1b 2 mg mL^{-1}	NG-3b 2 mg mL^{-1}	Deionized water	SP 10 µg mL^{-1}	NY 1 mg mL^{-1}
S. aureus (ATCC 21824)	5	5	5	5	5	24	5
E. coli (ATCC 23523)	5	5.5	13.5	18	5	30	5
C. albicans (NCYC 106)	5	5	5	5	5	5	27

The zone diameter recorded is zone of inhibition + size of cup (zone of inhibition +5)mm, CE = Crude ethanolic extract; ET = Ethyl acetate fraction, SP =Streptomycin, NY =Nystatin, NCYC = National Collection of Yeast Cultures, UK, ATCC = American Type Culture Collection**,** Washington, DC, USA.

Ions at 181 (7.81 %), 101 (8. 28 %) and 94 (21.45 %) shows the removal of phenyl and phenyl, ethoxy, ethyl and oximino moieties respectively from **NG-3b**. The most abundant ion (base peak) at 58 (100 %) and the fragment at 55 (32.75 %) indicate the excision of phenyl, ethoxy and oximino from the matrix.

The IR spectrum of the compound shows absorptions at 756, 758, 856, 859, 1047, 1048, 1606, 1609, 2219 and 3471 cm^{-1} which represent alkyl substitutions, ether linkage, aromatic C=C, -C=N and –OH absorptions, respectively.

It is pertinent to note that the isolation of **NG-1b** could be due to the partial esterification of cinnamic acid in ethanol during extraction. Also, **NG-1b** has been reportedly isolated from the rhizomes of *Kaempfera galanga* (L.).

This compound has been found to inhibit monoamine oxidase and possesses vasorelaxation activity.[8-10] However, a closer examination of the chemical structure of **NG-3b** shows a carbazole-like skeleton which has been found in studies to be important biogenetic precursors in phytochemistry.[12] Furthermore, this compound has been obtained from *Lepidium sativum* using GC-MS, and FTIR spectrometry.[11]

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 displayed in table 1 show that the crude extract, ethyl acetate fraction, **NG-1b** and **NG-3b** were inactive against S. *aureus* and *C. albicans*. Surprisingly, the two compounds were remarkably bacteriostatic against *E. coli*. However, it is imperative to indicate that **NG-3b** was more suppressive of the bacterium than **NG-1b**. This 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 in order because 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.[13]

Conclusion

The isolation of these compounds is being reported for the first time from the plant. Hence, NG-1band NG-3bare equally expected to serve as chemotaxonomic markers for this species and the genus, *Pycnanthus* in general. Furthermore, the results of the antimicrobial screening partly lend some justification for the use of this plant especially in the treatment /management of bacterial infections. However, the two compounds will be tested against other bacterial and fungal strains with the aim of obtaining improved activity.

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