African Journal of Pharmacology and Therapeutics Vol. 6 No. 2 Pages 83-87, 2017
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Research Article

Ethyl linalool and diethyl phthalate from *Pycnanthus angolensis* (Welw.) Warb.

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Background: *Pycnanthus angolensis* (Welw.) Warb**.** belongs to the family, Myristiceae. Extracts of the plant are used in African ethno-medicine to treat diabetes, lumbago, wounds, arthritis, anemia, mouth-thrush, scabies, infertility and skin-fungal infections amongst many others. Flavonoids, terpenes, fatty acid derivatives and quinones had previously been isolated from different organs of the plant. Before now, very scanty literature exits on organic fractions from where specific compounds had been obtained.

Objectives: To study the chemical and biological parameters of the ethyl acetate fraction obtained fractionation of crude ethanol extract of the leaves of *Pycnanthus angolensis*.

Methodology: The leaves were to be extracted cold with 50 % ethanol and the obtained aqueous crude extract partitioned with ethyl acetate. Furthermore, the ethyl acetate fraction was to be subjected to silica gel column chromatography and the isolated compounds screened for both antibacterial and antifungal activities using the microbes namely, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*.

Results: Two isolates coded **NG-2** (pale yellow compound; 62 mg; R_f (0.53); $[\alpha]_D^{20}$ (+3º); $[n]_D^{20}$ (1.4009) and **NG-4c** (off-white compound; 36 mg, R_f (0.24); $[\alpha]_D^{20}$ (0º); $[n]_D^{20}$ (1.5006) whose identities have been revealed to be 3-ethoxy-3, 7-dimethyl-1, 6-octadiene (ethyl linalool) and diethyl phthalate (1, 2-benzenedicarboxylic acid diethyl ester) respectively using the MS and IR spectral techniques. Both **NG-2** and **NG-4c** were strongly bacteriostatic against *E. coli*, but recorded no activity against *S. aureus* and *C. albicans*.

Conclusion: The isolation of the two compounds is being reported for the first time from the ethyl acetate fraction of the plant. Hence, **NG-2** and **NG-4c** would serve as chemotaxonomic markers for this species and the genus, *Pycnanthus* in general.

Keywords: *Pycnanthus angolensis;* fraction; ethyl linalool; diethyl phthalate: antimicrobial

Received: March, 2017
Published: June, 2017

1. Introduction

Pycnanthus angolensis (Welw.) Warb**.** syn*. P. kombo* (known as African or wild or false nutmeg, boxboard or cardboard) was originally native to the forest zones of West and Central Africa but now cultivated in and around the world (Hutchinson and Dalziel, 1954). Different preparations of the plant are employed in diverse African folklores to treat chest infections, diabetes, lumbago, wounds, arthritis, anemia, mouth-thrush, malaria, leprosy, toothache, infertility, sexually transmitted disease and skin-fungal infections (Keay et al*,* 1964; Etukudo, 2003; Hollist, 2008). The larvicidal and antitumor potentials of the plant have been studied (Oladimeji et al*,* 2012; Oladimeji et al*,* 2013) while reports of isolation of flavonoids and terpenes from the bark and roots and quinones from the leaves abound (Luo and Xiu, 1998). This present investigation aimed at isolating compound(s) from the ethyl acetate fraction which demonstrated the highest antimicrobial activity in a previous bioactivity-guided fractionation study of the plant (Oladimeji et al*,* 2006). 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.

2. Methods

2.1 Collection of Plant

The fresh leaves of *P. angolensis* were collected around the month of April, 2016 within the precinct of University of Uyo, Akwa Ibom State, Nigeria. The plant had previously been identified in a study by Oladimeji et al*,* 2006. Hence, a voucher specimen of the plant (**No. H049**) was deposited in the Herbarium Unit of the Faculty of Pharmacy. 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).

2.2 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 (R205D, Shensung BS & T, China). 250 g of dried crude extract was obtained and then stored in a desiccator (Monsorief, Scotland) 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.

Thereafter, 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 $(CH_3)_2$ CO: 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 $(CH_3)_2$ CO: 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 separately clean up the semi-pure residues 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).

2.3 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). The refractive indices and optical rotation were obtained using WAY-15 Abbe refractometer (England) and ADP-220 Bellingham Stanley polarimeter (England) respectively. The refractometer and polarimeter were initially zeroed and the refractive indices and optical rotation were measured at the wavelength (λ) of Na-D line (589.3 nm) and at 20 ^{0}C.

2.4 Antimicrobial Tests

The micro-organisms used in this study were limited to three viz: one gram (+), gram (-) and 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 (Gibson and Khoury, 1986; Murray et al*,* 1995). These clinical microbes were then refrigerated at -5 ^{0}C at the Microbiology and Parasitology Unit, Faculty of Pharmacy prior to use. 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 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 diameter of approximately 5 millimeters. The wells were equidistant from each other and the edge of the plate (Washington, 1995; NCCLS, 2003). 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), 1mg mL^{-1} of nystatin (Gemini Drugs, Nigeria) and deionized water were introduced into separate wells as positive and negative controls respectively (Oladimeji, 2012; Oladimeji and Igboasoiyi, 2014; Oladimeji and Udom, 2014; Oladimeji and Johnson, 2015). 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 ^{0}C for 24 to 48 h. Zones of inhibition were measured in millimeters (mm) with the aid of a ruler.

3. Results

Compound characterization

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 $[M\text{-}2H]^+$ (0.70 %), 166 $[M\text{-}CH_3\text{-}1H]^+$ (4.05 %), 150 $[M\text{-}2CH_3\text{-}2H]^+$ (3.30 %), 137 $[M\text{-}3CH_3]^+$ (3.20 %), 121 $[M\text{-}OC_2H_5\text{-}CH_3\text{-}1]^+$ (41.01 %), 107 $[M\text{-}OC_2H_5\text{-}2CH_3]^+$ (8.04 %), 96 $[M\text{-}OC_2H_5\text{-}3CH_3\text{+}4]^+$ (51.30 %), 71 $[M\text{-}OC_2H_5\text{-}3CH_3\text{-}21]^+$ (90.13 %), 69 $[M\text{-}OC_2H_5\text{-}3CH_3\text{-}23]^+$ (30.07 %) and 43 $[M\text{-}139]^+$ (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 $[M\text{-}C_2H_5 +1]^+$ (3.30 %), 177 $[M\text{-}OC_2H_5]^+$ (37.73 %), 164 $[M\text{-}OC_2H_5\text{-}CH_3\text{-}2]^+$ (4.06 %), 149 $[M\text{-}OC_2H_5\text{-}2CH_3 + 2]^+$ (100 %) 132 $[M\text{-}2OC_2H_5]^+$ (5.26 %), 121 $[M\text{-}2OC_2H_5\text{-}11]^+$ (6.08 %), 105 $[M\text{-}2OC_2H_5\text{-}CO\text{-}1]^+$ (7.31 %), 93 $[M\text{-}2OC_2H_5\text{-}CO\text{-}11]^+$ (7.50 %), 78 $[M\text{-}2OC_2H_5\text{-}2CO +2]^+$ (23.05 %), 65 $[M\text{-}2C_2H_5O\text{-}2CO\text{-}11]^+$ (18.26 %) and 50 $[M\text{-}2OC_2H_5\text{-}2CO\text{-}25]^+$ (20.15 %); IR [FTIR] cm^{-1}: 932, (alkyl substitution), 1072 (-C-O-C), (1602) Ar (-C=C) and 1721 (-C=O).

The determination of physical constants such as optical rotation and refractive index is used in the qualitative and quantitative analyses of substances. Also, these parameters and others are employed to confirm the purity, identity, integrity of active substances and as well as monitor the progress of reactions (Olaniyi, 1989; Olaniyi and Ogungbamila, 1991; Olaniyi, 2000).

Consequently, the refractive index of **NG-2** and **NG-4c** was separately determined at the wavelength (λ) of Na-D light (589.3 nm) and at temperature of 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. Furthermore, refractive index can be used to monitor the progress of chromatographic separation by measuring the refractive indices of the effluent solvents employed. **NG-2** and **NG-4c** recorded refractive index of 1.4009 and 1.5006 respectively and these values are consistent with those in literature (1.4006 and 1.5002). In addition, **NG-2** showed an optical rotation of +3 indicating dextrorotation while **NG-4c** demonstrated a rotation of zero showing neither dextrorotation nor levorotation. The chemical structures of the compounds were established by a combination of spectroscopic techniques as highlighted above. The obtained data were matched with those in the library data of organic compounds (Lopez-Avila, 1987), hence, **NG-2** and **NG-4c** have been identified to be 3-ethoxy-3,7-dimethyl-1, 6-octadiene (ethyl linalool) and diethyl phthalate (1, 2-benzenedicarboxylic acid diethyl ester) respectively as presented in **Figures 1**.

Due to the nature of the matrices, many fragmented peaks appeared in the MS spectra of the compounds. In the mass spectrum of **NG-2,** those that are easily identifiable include; $[M]^+$ 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** while 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.

Equally, **NG-4c** showed numerous peaks in its MS matrix but those that could readily be identified include $[M]^+$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.

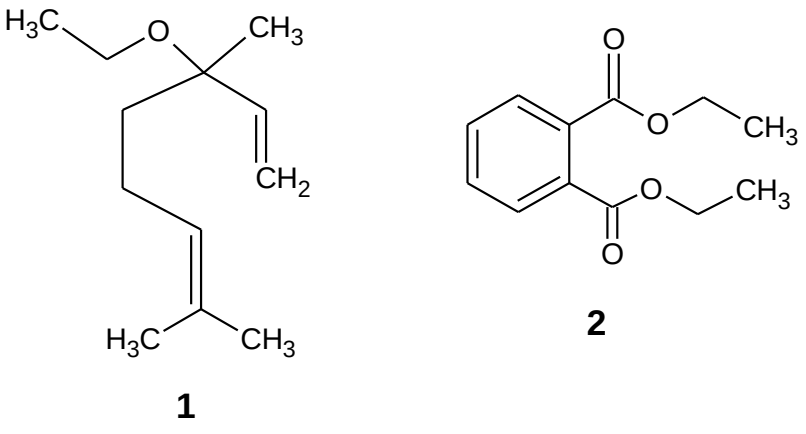

Figure 1: 3-ethoxy-3, 7-dimethyl-1, 6-octadiene (ethyl linalool) (**1**) and Diethyl phthalate (1, 2-benzenedicarboxylic acid diethyl ester) (**2**)

Table 1: Results of antimicrobial screening of crude extract, ethyl acetate fraction, **NG-2** and **NG-4c** at different concentrations on test microbes in water

Test microbe	CE (20 mg/mL)	ET (10 mg/mL)	NG-2 (2 mg/mL)	NG-4c (2 mg/mL)	Deionized water	SP (10 μg/mL)	NY (1 mg/mL)
S. aureus (ATCC 21824)	5	5	5	5	5	26	5
E. coli (ATCC 23523)	5	5	17	15	5	31	5
C. albicans (NCYC 106)	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.

3.4 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 the **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 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 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 (Brown, 1975). It is instructive to mention that derivatization studies are currently on-going in our laboratories with the aim of improving on the observed activity.

4.0 Conclusion

The isolation of the two compounds is being reported for the first time from the ethyl acetate fraction of the plant. Hence, **NG-2** and **NG-4c** are expected to serve as chemotaxonomic markers for this species and the genus, *Pycnanthus* in general. Furthermore, the results of the antimicrobial sensitivity tests partly lends some credence to the use of this plant especially in the treatment /management of bacterial disease. However, the two compounds will be further screened against other bacterial and fungal strains with the aim of obtaining better antimicrobial activity.

Conflict of Interest declaration

The authors declare no conflict of interest.

Acknowledgements

The authors thank the Shimadzu Training Centre for Analytical Instruments (STC) Lagos, Nigeria for the assistance rendered in obtaining the spectra of the compounds. The role of E. Akpan, Principal Technologist, Pharmaceutical Microbiology Unit, Faculty of Pharmacy, University of Uyo, Uyo, Nigeria in the conduct of the antimicrobial screening is hugely appreciated.

References

Brown, MR (1975). A question of resistance. *Pharm. J.* **215:** 239-242.

Etukudo, I (2003). Convectional and traditional uses of plants. Ethno-botany. Verdict press, Uyo, pp. 11-21.

Gibson, L, and Khoury, J (1986). Storage and survival of bacteria by ultra-freeze. *Lett. Applied Microbiol***. 3:** 127-129.

Hollist, NO (2008). A collection of traditional Yoruba oral and dental medicaments. Royal press, Ibadan, pp. 54-57.

Hutchinson, J, and Dalziel, JM. (1954). Flora of west tropical Africa. Vol. I, Part I. Crown agent for overseas governments and administrations, London, p. 121.

Keay, RWJ, Onochie, CF, and Stanfield, DP (1964). Nigerian trees Vol. I. Nigerian national press limited, Lagos, pp. 43-44.

Lopez-Avila, V (1987). Stability of organic compounds with extracted matrices with excitable energy. *Org. Mass Spec.,* **22**: 557.

Luo, J, and Xiu, R (1998). Anti-diabetic and anti-fungal potential of isolated terpenoidquinones from *Pycnanthus angolensis* bark. *J. Pharmacol. Exp. Ther.* **288:** 529-534.

Murray, P, Baron, E, Pfaller, M, Tenover, F, and Yolken, R (1995). Manual of Clinical Microbiology. American Society of Microbiology Press, p. 973.

NCCLS (2003). Performance standard for antimicrobial susceptibility test. 8th edition, Approved standard, p. 130.

Oladimeji, OH, Ubulom, PME, Igboasoiyi, AC, Ndukwe, K, and Nia, R (2006). Some biological activities of *Pycnanthus angolensis* (Welw.) Warb. *J. Pharm. Biores.* **3:** 49-55.

Oladimeji, HO, Ani, L, and Nyong, EE (2012). Potential larvicides in Nigerian herbal recipes. *Int. J. Pharm. Sci. Res.* **3:** 3783-3787.

Oladimeji, HO, Ubulom, PME, and Olugbade TA (2013). Cytotoxicity studies on some Nigerian medicinal plants. *Advance Res. Pharm.Biol.* **3:** 403-407.

Oladimeji, HO, and Igboasoiyi**,** AC (2014). Isolation, characterization and antimicrobial analysis of ethyl gallate and pyrogallol from *Acalypha wilkesiana var. lace-acalypha* (Muell & Arg.). *Afri. J. Pharmacol. Ther.* **3:** 79- 84.

Oladimeji, HO, and Udom, FI (2014). D-arabino-hex-1-enitol from the inactive fraction of *Acalypha wilkesiana var. lace-acalypha* (Muell & Arg.). *Eur. Chem. Bull.* **3:** 1060-1063.

Oladimeji, HO, and Johnson, EC (2015). Glucolipid from the ethyl acetate fraction of *Acalypha wilkesiana var. lace-acalypha* (Muell & Arg.). *J. Pharm. Biores.* **12:** 48-53.

Olaniyi, AA (1989). Essential medicinal chemistry. 1st edition. Shaneson C. I. Limited, Ibadan, pp. 137-154.

Olaniyi, AA and Ogungbamila, FO (1991). Experimental pharmaceutical chemistry. Shaneson C. I. Limited, Ibadan, pp. 78-79.

Olaniyi, AA (2000). Principles of quality assurance and pharmaceutical analysis. Mosuro publishers, Ibadan, pp. 151-158, 216-217, 264-269 and 443-457.

Washington J (1995). The agar diffusion method. In**:** Manual of clinical microbiology. 4th edition, American Society of Microbiology Press, pp. 971-973.