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**ISOLATION AND CHARACTERIZATION OF DI-(1-HEXEN-5-YL) PHTHALATE
AND MONOETHYL PHTHALATE FROM THE ETHYL ACETATE EXTRACT OF
UNRIPE FRUITS OF *Nauclea latifolia***

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ABSTRACT

The aim of this study is to purify, identify and characterize some of the phytoconstituents in the ethyl acetate extract of the unripe fruits of *Nauclea latifolia* (Family: Rubiaceae). The extract was subjected to a series of flash column chromatography, followed by purification with sephadex LH-20 to afford two phthalate derivatives that were subjected to physical, chemical and spectral identification by IR, UV, ¹H-NMR, ¹³C-NMR, DEPT-135, GC-MS and literature search. The compounds were identified as Di-(1-hexen-5-yl) phthalate (63% yield) and Monoethyl phthalate (34% yield). This is the first report of the isolation of these compounds from the unripe fruits of *N. latifolia*, although other phthalates have been reported.

Keywords: *Nauclea latifolia*, Unripe Fruits, Ethyl Acetate, Spectroscopic Analysis, Di-(1-Hexen-5-yl) Phthalate, Monoethyl Phthalate

INTRODUCTION

The use of traditional medicines as therapeutic agents for the maintenance of good health in most developing countries has been widely observed [1]. Plants continue to play a major role in the maintenance and improvement of human health for centuries in both developing and developed countries of

the world. These plants provide structural diversity in producing natural products and this diversity makes them an interesting source for the exploration of new bioactive lead compounds. The search for such lead compounds is most often based on claims from ethno medicine which prompts

phytochemical investigation of such plants [2]. It is therefore pertinent for us to keep investigating such plants for their bioactive compounds. *Nauclea latifolia* Smth (Family: Rubiaceae) is a small tree made of fruits which has several brownish seeds embedded within it and surrounded by a pink, edible, sweet-sour pulp. The fruits are usually red and fleshy when ripe, resembling hard strawberry and yellow when unripe [3]. Traditionally, dried fruits of the plant have been reported useful as an anti-dysenteric and anti-diarrhoeal [4]. The antibacterial activity of the fruits has also been reported [5]. The fruit is said to contain adequate amounts of vitamins A, B₁, B₂, C and E and therefore could be important in the prevention of night blindness [6]. Result of biochemical evaluation of the fruits of the plant revealed high values of calcium, phosphorous and magnesium and could be useful in the development of bones and teeth and could serve as an anti-clotting agent. The level of iron was reportedly high (1.8-6.7mg/100g), a range 2-5 times higher than that obtained in fruits like oranges and mangoes [7]. Phytochemical studies of the acetone portion of the ripe fruits and ethyl acetate portion of the unripe fruits led to the isolation and characterization of di-(2-ethylhexyl) phthalate for the first time from both fruits, with the

ripe fruits possessing higher quantity of the isolate than the ripe [8, 9]. In view of the absence of much information on the phytoconstituents of the fruits, the ethyl acetate extract of the unripe fruits of *N. latifolia* was further screened. In this paper, therefore, the isolation and structure elucidation of two phthalates from the ethyl acetate extract of the unripe fruits of *N. latifolia* is been described.

MATERIALS AND METHODS

Sample Collection

The unripe fruits of *N. latifolia* were collected from a farmland in Maikunkele area of Bosso Local Government area, Minna, Niger State, Nigeria, in the month of January, 2013. The fruits were authenticated by Mallam Gallah, of the Herbarium section of the Department of Biological Sciences, Ahmadu Bello University, Zaria and a voucher specimen was deposited.

Extraction Procedures

Air-dried, pounded and sieved unripe fruits of *N. latifolia* (500g) were exhaustively extracted with ethyl acetate by continuous maceration using a sonic bath. The resulting solution was filtered, concentrated in vacuo and further dried over a water bath. Extract was labeled 'A'

Fractionation of Crude Extract 'A'

Twenty two grams of portion 'A' was fractionated using vacuum liquid

chromatography. Silica gel (60-120 mesh, 700g) was used as the stationary phase, while varying proportions of increasing polarity of petroleum ether-chloroform; chloroform-ethyl acetate and ethyl acetate-acetone was used as the mobile phase. Similar fractions were pooled using TLC, yielding nine major fractions, labeled 'A1' to 'A9'

Further Purification of Fraction 'A3'

Three grams of fraction 'A3' was subjected to further purification by flash column chromatography using silica gel (mesh 60-120, 90g) and varying proportions of chloroform: ethyl acetate as stationary and mobile phases respectively. Similar sub-fractions were pooled using TLC, yielding four major sub-fractions, labeled 'A3-a' to 'A3-d'

Further Purification of Sub-Fraction 'A3-a'

Purification of sub-fraction 'A3-a' (450mg) using flash chromatography (silica gel mesh 230-400, 30g) and increasing polarity of chloroform: ethyl acetate gave rise to a mixture that showed two distinct spots on TLC from eluting solvent of chloroform: ethyl acetate (19:1).

Further Purification of Sub-Fraction 'A3-a'

Two hundred and fifty milligrams (glittery yellow flakes) of 'A3-a' was subjected to further purification by column

chromatography using sephadex LH-20 and a mixture of chloroform: methanol (1:1) as stationary and mobile phases respectively to yield two distinct compounds as revealed by GC-MS. They were labeled 'A3-a1' and 'A3-a2'

Characterization of Compounds 'A3-a1' and 'A3-a2'

Chromatograms were viewed under UV (254 and 366nm), iodine crystals and sprayed with 50% sulphuric acid reagent.

Melting point was uncorrected. FTIR (Spectrolab MB3000) and UV spectra (T60 UV-Visible spectrophotometer) were taken in CHCl_3 ; GC-MS was recorded using GCMS-QP 2010 plus Shimadzu. $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and DEPT-135 spectra were taken in CDCl_3 on Bruker ACQ 400 Avance spectrometer operating at 400MHz.

RESULTS AND DISCUSSION

Physical and Spectral Characterization for Compound 'A3-a1'

A colorless viscous oil (63% yield), slight sweet smell, soluble in chloroform, acetone, methanol; insoluble in water, indicating that it is a mid-polar compound. Spot on TLC (R_f 0.47) was UV active (pink, 254nm and blue, 366nm), golden brown with I_2 crystals; no color with sunlight nor 50% H_2SO_4 . Its molecular formula and weight by GC-MS was found to be $\text{C}_{20}\text{H}_{26}\text{O}_4$ and $330\text{g}\text{mol}^{-1}$

respectively, while its melting point was 187-192°C, indicating that it is a slightly high melting compound.

UV (CHCl_3) spectrum indicated characteristic peaks at λ max (nm): 181 (sp^2 hybridized chromophore), 187 (benzene chromophore) and 274 (presence of carbonyl group)

IR (CHCl_3) spectrum indicated characteristic peaks at ν max (cm^{-1}): 1212 (acetate stretch of an ester), 1600 (benzene ring, broad), 1738 (carbonyl stretch of an ester), 2900-2800 (C-H, stretch) and 3068-3060 (C-H stretch of sp^2 carbon atoms)

$^1\text{H-NMR}$ (400 MHz, CDCl_3) spectrum indicated characteristic peaks at δ (ppm): 7.75-7.72 (dd; H-2 and H-5), 7.68-7.61 (dd; H-3 and H-4), 5.31-4.76 (quintet; H-6'/6''; H-7'/H-7'' doublet), 4.27-4.24 (sextet; H-3'/H-3''), 2.52-2.34 (quartet; H-5'/H-5''), 1.5-1.4 (quartet; H-4'/H-4'') and 0.97-0.93 (doublet; H-8'/H-8''). Anisotropic effect produced by the π system in compound 1 influenced the chemical shift of nearby protons, so that allylic hydrogen atoms are shifted downfield [10].

$^{13}\text{C-NMR}$ (400 MHz, CDCl_3) spectrum indicated characteristic peaks at δ (ppm): 169.9 (singlet; carbonyl; C-1' & C-1''), 135.3 (singlet; C-1 & C-6), 132.8 (doublet; C-3 & C-4), 130.6 (doublet; C-2 & C-5), 124.6 (doublet; C-6 & C-6''); 119.8 (triplet; C-7 &

C-7''), 72.1 (doublet; C-3' & C-3''), 34.7 (triplet; C-5' & C-5''), 27.8 (triplet; C-4' & C-4'') and 16.3 (quartet; C-8' & C-8''). Carbon-13 NMR revealed a total of ten carbon atoms with the number of protons directly attached to each carbon/multiplicity of the peaks (off-resonance proton-decoupled carbon-13 spectra) shown as singlet (s), doublet (d), triplet (t) and quartet (q) spectra.

DEPT-135 (400 MHz, CDCl_3) spectrum indicated characteristic peaks at δ (ppm): 169.9 (quaternary; C-1' & C-1''), 135.3 (quaternary; C-1 & C-6), 132.8 (methine; C-3 & C-4), 130.6 (methine; C-2 & C-5), 124.6 (methine; C-6' & C-6''), 119.8 (methylene; C-7' & C-7''), 72.1 (methine; C-3' & C-3''), 34.7 (methylene; C-5' & C-5''), 27.8 (methylene; C-4' & C-4'') and (methyl; C-8' & C-8''). Distortion less enhancement by polarization at pulse 135° revealed the compound is made up of a total of two quaternary carbons, three methines, three methylenes and one methyl carbon atoms.

GC-MS (CHCl_3) spectrum indicated characteristic peaks of fragment ions (m/z) at : 330; [M, $\text{C}_{20}\text{H}_{26}\text{O}_4$], 149; [$\text{C}_8\text{H}_5\text{O}_3$] $^+$, 132; [M - 2 x OC_2H_{11}] $^-$, 122; [$\text{C}_7\text{H}_6\text{O}_2$] $^+$, 121; [$\text{C}_7\text{H}_5\text{O}_2$] $^-$, 105; [$\text{C}_7\text{H}_5\text{O}$] $^-$, 93; [$\text{C}_6\text{H}_5\text{O}$] $^+$, 76; [C_6H_4] $^-$, 65; [C_5H_5] $^+$, 50; [C_3H_4] $^+$, 39; [C_3H_3] $^+$ and 27; [C_2H_3] $^+$. The GC-MS fragmentation patterns revealed compound

'A3-a1' to be a phthalate with characteristic peaks at m/z 149 (base peak, a phthalic acid ester), 122 (McLafferty re-arrangement) 104/105 (common peak for esters) and 76 (aromatic ketone) [11]. It revealed 20 carbon atoms (although carbon-13 revealed 10) an indication that the compound consisted of two identical parts, which are mirror images of each other [12]. Correlation of obtained physical characteristics and spectral data with literature revealed compound 'A3-a1' to be Di-(1-hexen-5-yl) phthalate as shown in Figure 1

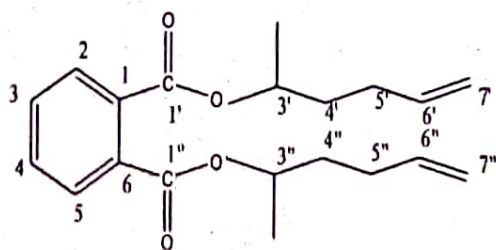


Figure 1: Di-(1-hexen-5-yl) Phthalate/Phthalic Acid, di (1-Hexen-5-yl) Ester/Benzene-1, 2-Dicarboxylic Acid Bis (1-Methylpent-4-Enyl) Ester/Bis (hex-5-en-2-yl) Benzene-1, 2-Dicarboxylate

Physical and Spectral Characterization for Compound 'A3-a2'

Light brown amorphous powder (34% yield), soluble in chloroform, acetone, ethyl acetate, methanol; slightly soluble in hexane and insoluble in water, indicating that it is also a mid-polar compound. Spot on TLC (R_f 0.85) was UV active (pink, 254nm and blue, 366nm), golden brown with I_2 crystals; no color with sunlight nor 50% H_2SO_4 . Its molecular formula and weight by GC-MS was found to be $C_{10}H_{10}O_4$ and 194.2 g mol^{-1}

respectively, while its melting point was 83 - 86°C, indicating it is not a too low melting compound.

UV ($CHCl_3$) spectrum indicated characteristic peaks at λ max (nm): 227 (benzene chromophore bearing a COOH group)

IR ($CHCl_3$) spectrum indicated characteristic peaks at ν max (cm^{-1}): 2900-2450 (O-H stretch of COOH), 2849 (CH_2 group) and 2871 (CH_3 group)

1H -NMR (400 MHz, $CDCl_3$) spectrum indicated characteristic peaks at δ (ppm): 7.98 (doublet; H-2), 7.72 (doublet; H-5), 7.48 (triplet; H-3), 7.32 (triplet, H-4), 4.17 (quartet; H-3'), 1.92 (triplet; H-4'). The presence of a deshielded (low field) proton at δ 10.8 is indicative of a carboxylic acid proton, while, δ 7.98 indicates a COOH group neighboring to an aryl group.

^{13}C -NMR (400 MHz, $CDCl_3$) spectrum indicated characteristic peaks at δ (ppm): 167.7 (singlet; C-1'), 168.9 (singlet; C-1''); 133.5 (singlet; C-1), 133.1 (singlet; C-6), 130.2 (doublet; C-2), 129.6 (doublet; C-3), 129.1 (doublet; C-4), 128.7 (doublet; C-5), 63.6 (triplet; C-3') and 14.2 (quartet, C-4'). Carbon-13 NMR also revealed the compound is made up of a total of ten carbon atoms (10 peaks).

DEPT-135 (400 MHz, $CDCl_3$) spectrum indicated characteristic peaks at δ (ppm):

167.7 (quaternary; C-1'), 168.9 (quaternary; C-1''); 133.5 (quaternary; C-1), 133.1 (quaternary; C-6), 130.2 (methine; C-2), 129.6 (methine; C-3), 129.1 (methine; C-4), 128.7 (methine; C-5), 63.6 (methylene; C-3') and 14.2 (methyl, C-4'). Compound is made up of four quaternary carbons, three methines, one methylene and one methyl carbon atoms.

GC-MS (CHCl_3) spectrum indicated characteristic peaks of fragment ions (m/z) at: 194; $[\text{M}, \text{C}_{10}\text{H}_{10}\text{O}_4]$, 149; $[\text{C}_8\text{H}_5\text{O}_3]^+$, 122; $[\text{C}_7\text{H}_6\text{O}_2]^+$, 104; $[\text{C}_7\text{H}_4\text{O}]^+$, 93; $[\text{C}_6\text{H}_5\text{O}]^+$, 76; $[\text{C}_6\text{H}_4]^+$, 65; $[\text{C}_5\text{H}_5]^+$, 50; $[\text{C}_3\text{H}_4]^+$, 31; $[\text{CH}_3\text{O}]^+$ and 27; $[\text{C}_2\text{H}_3]^+$. GC-MS of compound 'A3-a2' also revealed it is a phthalate, just like compound 'A3-a1'. Both its GC-MS and carbon-13 spectra revealed exactly 10 carbon atoms, showing that it did not consist of two identical parts. Correlation of obtained physical characteristics and spectral data with literature revealed compound 'A3-a2' to be Monoethyl phthalate (MEP) as shown in Figure 2.

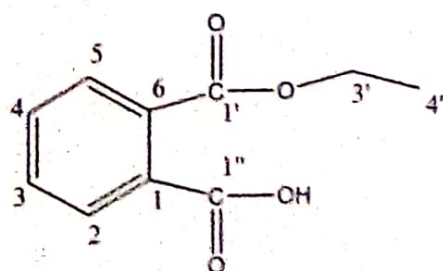


Figure 2: Monoethyl phthalate (MEP) / 1, 2-Benzenecarboxylic acid, monoethyl ester/Phthalic acid, ethyl ester/ Phthalic acid, monoethyl ester

Generally, phthalates are a family of aromatic homo monocyclic compounds or derivatives of benzoic acid or benzoic di esters with the ester groups ortho to each other. They are largely hydrophobic with low volatility, having a wide range of application in cosmetics, solvents, adhesives, paints, building materials, children's toys, insecticides and plasticizers [13]. Exposure to phthalates has been reported to have health implications [14, 15, 16]. The presence of some of these phthalates in vegetables and fruits has been reported [17]. Human diet had earlier been reported as one of the major routes of exposure to these phthalates, although they are short-lived in human bodies [13]. Studies have shown that these phthalate esters (PE) which are naturally produced extracellularly by micro organisms, such as bacteria, fungi and yeasts [18] have been detected in bacteria, plants and the fatty acid fractions of certain species of marine macroalgae [19, 20, 21]. The presence of monoethyl phthalate (MEP) in the fruits of *N. latifolia* is not surprising. MEP is a primary metabolite of di-(2-ethylhexyl) phthalate (DEHP) [22] and DEHP had earlier been isolated from the fruits [8, 9].

CONCLUSION

Two phthalates, di-(1-hexen-5-yl) phthalate (major) and monoethyl phthalate (minor) have

been isolated and characterized from the ethyl acetate extract of the unripe fruits of *N. latifolia* for the first time. Further work will be directed towards isolating and characterizing more biologically-active compounds from the ripe and unripe fruits of *Nauclea latifolia*.

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