

Antibacterial Activity of 1,2-Benzenedicarboxylic Acid, Dioctyl Ester Isolated from the Ethyl Acetate Soluble Sub-portion of the unripe Fruits of *Nauclea latifolia*

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ABSTRACT

A phytochemical and antibacterial study of the ethyl acetate soluble sub-portion of the methanol extract of the unripe fruits of Nauclea latifolia (Family Rubiaceae) its fractions and sub-fractions led to the isolation of 1, 2-benzenedicarboxylic acid, dioctyl ester for the first time from the unripe fruits of the plant. The structure of the viscous golden yellow oil was elucidated using physical, chemical, literature search and spectroscopic identification. The isolated compound (Me2-b1 at 100µg/ml) displayed moderate inhibitory activity against Gram positive organisms tested, similar to that exhibited by chloramphenicol against the organisms. The crude ethyl acetate soluble sub-portion Me, at 50mg/ml exhibited significant broad spectrum activity, while one of its fractions (Me2 at 20mg/ml) and sub-fraction (Me2-b at 20mg/ml) were moderately active against most of the test bacterial strains.

Key words: *Nauclea latifolia* unripe fruits, ethyl acetate soluble sub-portion, antibacterial, 1, 2-benzenedicarboxylic acid, dioctyl ester.

INTRODUCTION

The continual search for, and the interest in natural plant products for use as medicines has for a long time acted as the catalyst for exploring these plants for their various secondary metabolites. The bioactivities of such plants make natural products an important source for the discovery of new drugs that are highly effective with low cost and toxicity. Also, the development of resistance of micro-organisms to conventional drugs has made the search for new drugs inevitable.

The plant, *Nauclea latifolia* Smth (Family: Rubiaceae) has over the years become a plant of study for us. This is because the plant had earlier been described as one of the plants with promising anti-infective activity/ interesting biological activity¹. The plant is a small tree of about 7m high that could grow up to 35m in closed forests. Its bark is glabrous, with a reddish slash; leaves are glabrous, opposite and glossy green. The fruits which are usually fleshy are red when ripe, resembling hard strawberry and yellow when unripe. Embedded within the fruits are numerous small brownish seeds surrounded by a pink, edible, sweet-sour pulp². The dried fruit is used traditionally in the treatment of dysentery, diarrhea and piles¹, while its extract has been shown to be active against Human Immune Deficiency Virus³. Non-medicinally, the plant is regarded as a source of food in Sudan and Northern Nigeria^{4,5}. The fruit is reportedly rich in vitamin C and this has made it a good source of fruit juice⁶. Earlier work revealed that the petroleum ether extract and sub-portions of the unripe fruits exhibited better antibacterial efficacy against some bacterial strains than that of the ripe fruits⁷. Further investigation carried out on the acetone-soluble portion of the methanolic extract of the ripe fruits of the plant led to the isolation of di-(2-ethylhexyl) phthalate, a compound that displayed moderate antibacterial activity at 100µg/ml against

Gram positive *B. subtilis* and *S. aureus*, an activity similar to that displayed by tetracycline (1mg/ml) against the same organisms⁸. We, therefore proceed to see if the same compound, with similar antibacterial efficacy could be isolated from the unripe fruits. This work, therefore, presents the result of the antibacterial activity-guided isolation of a phthalic acid derivative from the ethyl acetate soluble sub-portion of the unripe fruits of *N. latifolia* against some pathogenic bacteria in comparison with chloramphenicol.

MATERIALS AND METHODS

Collection of plant material

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, 2012. 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* (2kg) were exhaustively extracted with methanol by maceration at room temperature. The resulting solution was filtered and the filtrate concentrated in vacuo. Extract was further dried over a water bath and defatted with petroleum spirit (60-80°C) by maceration at room temperature for a week.

Defatted crude methanol extract, M, was re-extracted exhaustively with 200ml x 7 portions of ethyl acetate by maceration using a flask shaker. The resulting filtrate was concentrated, dried, weighed and coded ethyl acetate soluble sub-portion of defatted crude methanol extract of unripe fruits of *N. latifolia* (Me). Crude 'Me' was subjected to antibacterial screening in comparison with chloramphenicol (Figure 1).

Fractionation of ethyl acetate-soluble portion (Me)

Thirty grams of portion 'Me' was fractionated using vacuum liquid chromatography. Silica gel (60-120 mesh, 800g) 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 and subjected to antibacterial testing (Figure 2).

Further purification of fraction Me2.

Six grams of fraction Me2 was subjected to further purification by flash column chromatography using silica gel (mesh 60-120, 180g) and varying proportions of petroleum ether-chloroform as stationary and mobile phases respectively. Pooled sub-fractions were also subjected to antibacterial testing (Figure 3).

Further purification of sub-fraction Me2-b

Purification of sub-fraction Me2-b (350mg) using flash chromatography (silica gel mesh 230-400, 30g) and increasing polarity of hexane: chloroform gave rise to a mixture with a distinct spot.

Preparative TLC of sub-fraction Me2-b

A solution of Me2-b in chloroform was applied in form of a transverse band across the chromatographic plate (1mm), air-dried and developed in hexane: CHCl₃ (6:4). Major band was scrapped with the aid of a UV lamp and the resulting silica gel mixture triturated with acetone, filtered and concentrated in vacuo. This gave rise to a single spotted compound, coded Me2-b1 (Figure 4). It was also subjected to antibacterial testing in comparison with chloramphenicol (Figure 5).

Characterization of compound Me2-b1

Chromatograms were viewed under UV (254 and 366nm), iodine crystals and sprayed with 1% vanillin in sulphuric acid reagent.

Melting point was uncorrected. Rotation (Rudolph Autopol IV automatic polarimeter), FTIR (Spectrolab MB3000) and UV spectra (T60 UV-Visible spectrophotometer) were taken in CHCl₃; GC-MS was recorded using GCMS-QP 2010 plus Shimadzu. ¹H-NMR¹³, C-NMR and DEPT-135 spectra were taken in CDCl₃ on Bruker ACQ 400 Avance spectrometer operating at 400MHz.

Antibacterial testing

Bacterial cultures

The antibacterial activities of crude portion/fractions/sub-fractions/isolate were tested against overnight cultures of two *Gram* positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and four *Gram* negative bacteria (*Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumonia* and *Escherichia coli*). All organisms were obtained from the Department of Microbiology, Federal University of Technology, Minna, Nigeria. Organisms were standardized by sub culturing into nutrient broth at 37°C for 18h. Organisms were maintained in agar slants at 4°C and sub cultured 24h before use.

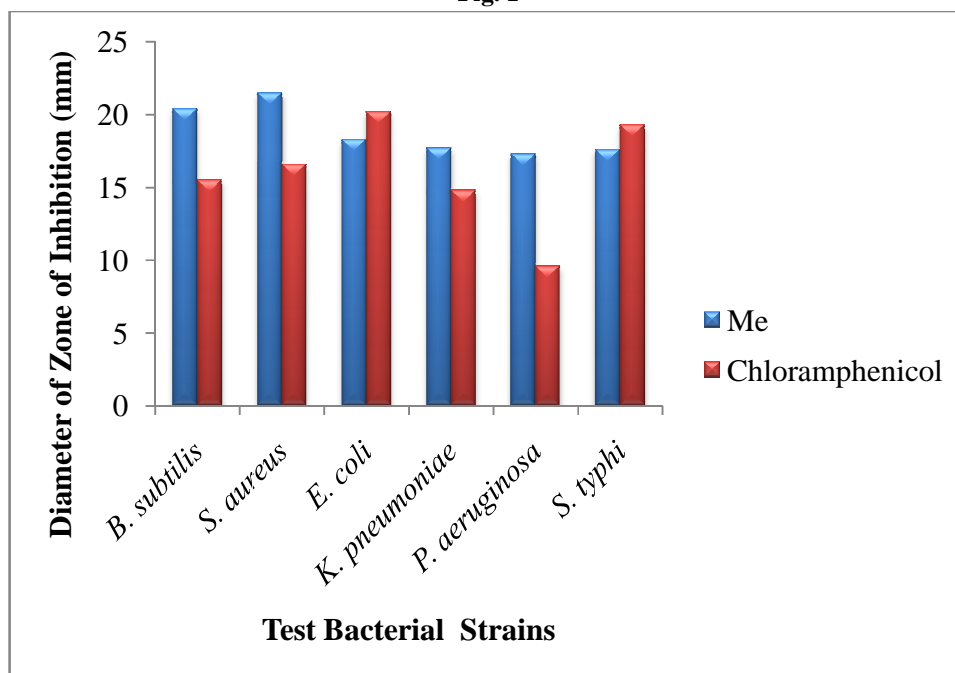
Bacterial susceptibility testing

The agar-well diffusion method was employed^{9,10}. Standardized inoculums containing 10⁶ cfu/ml 0.5 ml McFarland standards were evenly streaked onto the surface of sterile agar plates for each organism. 8mm wells were bored into the solidified agar using sterile cork borer at equidistant. 100µl of reconstituted Me (50mg/ml), Me2 (20mg/ml), Me2-b (20mg/ml), Me2-b1 (100µg/ml), positive control (Chloramphenicol, 1mg/ml) and negative control (methanol) was introduced into different wells individually with the aid of a Pasteur pipette. Agar plates were incubated aerobically at 37°C for 24h. Zones of inhibition around the wells were measured to the nearest millimeter using a meter rule. Experiments were carried out in duplicates. A test compound is considered 'active', when it has an inhibition zone of ≥ 14 mm¹¹.

RESULTS AND DISCUSSION

The crude ethyl acetate-soluble sub-portion of the unripe fruits of *N. latifolia* (Me) at 50mg/ml exhibited a broad spectrum better than that displayed by chloramphenicol at 1mg/ml against most of the test organisms (Figure 1).

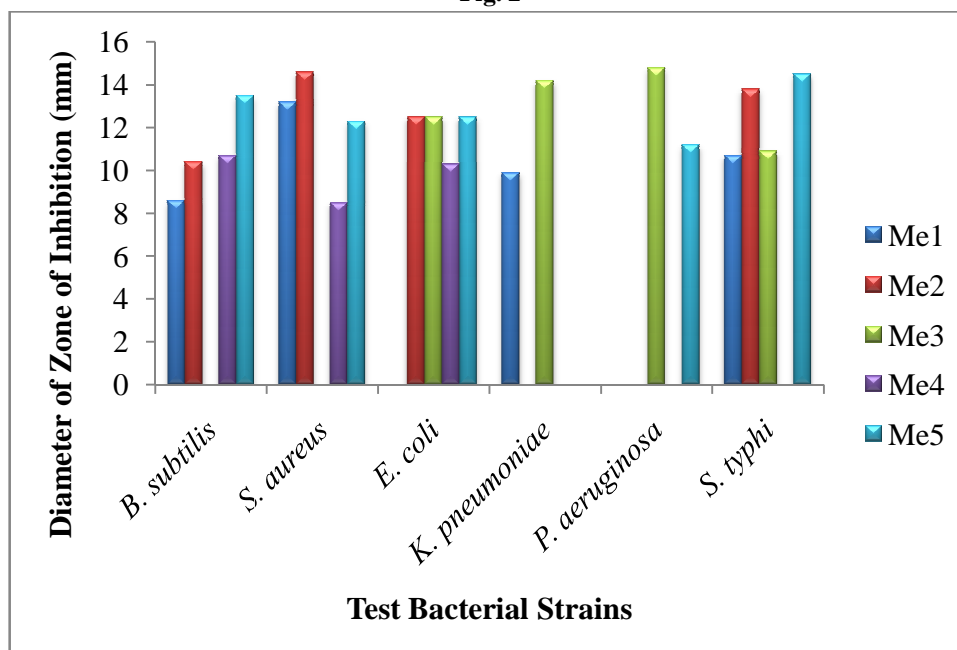
Fig. 1



Antibacterial activity of the crude ethyl acetate-soluble sub-portion, Me (50mg/ml) in comparison with chloramphenicol (1mg/ml) against test bacterial strains

Fractionation of the 'active Me' by vacuum liquid chromatography gave rise to 5 major fractions. Antibacterial activity of the 5 fractions at 20mg/ml (Figure 2) showed that they displayed moderate broad spectrum activity, an activity that was lesser than that displayed by the crude 'Me' at 50mg/ml against the test organisms. Sometimes, fractionation does not improve the antibacterial potency of a medicinal plant^{12,13}. Furthermore, the better biological activity of crude 'Me' probably reflects contribution from a number of constituents¹⁴.

Fig. 2

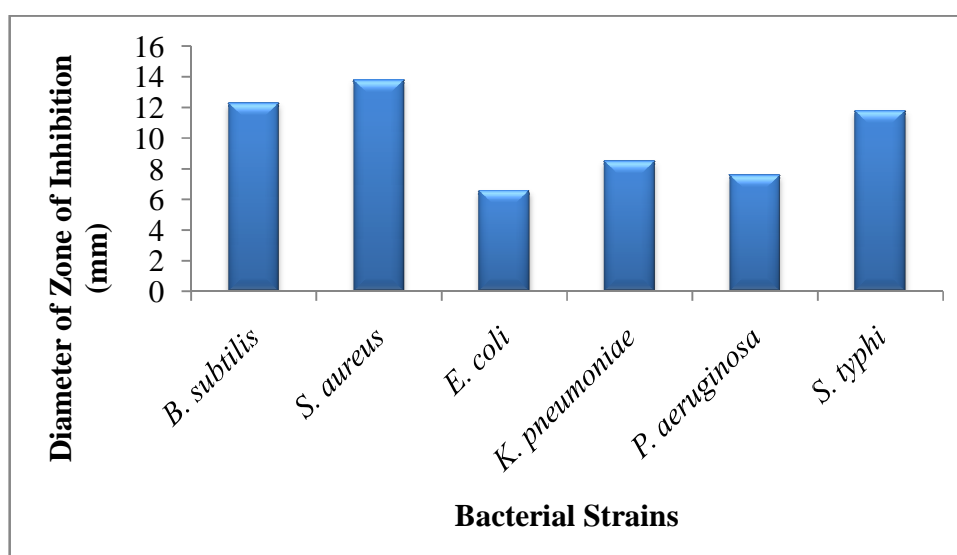


Antibacterial activity of the 5 fractions (20mg/ml each) obtained from VLC of crude 'Me' sub-portion against test bacterial strains.

Further fractionation of fraction Me2 (golden brown oil) on column chromatography gave rise to sub-fraction Me2-b (golden yellow oil) from solvent system petroleum ether: chloroform (9:1). Antibacterial activity of Me2-b at 20mg/ml showed that its activity did not differ significantly from that of fraction Me2 at 20mg/ml (Figure 3).

Further purification of the golden yellow oil by PTLC yielded a single spotted golden yellow oil (coded Me2-b1).

Fig. 3



Antibacterial activity of sub-fraction Me2-b at 20mg/ml against test bacterial strains

Characterization of Compound Me2-b1

Physical Characterization

A golden yellow viscous oil (27.4mg), slight odor, soluble in chloroform, acetone, methanol; insoluble in water, indicating that it is a mid-polar compound. Spot on TLC (petroleum ether: chloroform 1: 1) was

UV active (pink, 254nm and blue, 366nm), R_f (0.45, deep blue when sprayed with vanillin-sulphuric acid; golden brown with I_2 crystals; no color with sunlight), indicating that it is an aromatic compound^{15, 16}. Its molecular formula and weight by GC-MS was found to be $C_{24}H_{38}O_4$ and 390.56 respectively, while its melting point was $-55^\circ C$. Its specific rotation taken in $CHCl_3$ at $21^\circ C$ using D-line was found to be $[\alpha]^{21}_D$ (589nm) = 0.588, indicating that the molecule is only slightly chiral¹⁷

Spectral Characterization

UV λ max (nm): 275 (C-O, carbonyl)

IR ν max (cm^{-1}): 2900-2800 (C-H, aliphatic); 1732 (C-O, carbonyl); 1571 (aromatic ring); 1125 (C-O, acetate).

1H -NMR (ppm): 7.738-7.706 (dd; H-2 and H-5); 7.570-7.547 (dd; H-3 and H-4); 4.281-4.197 (septet; H-3' and H-3''); 1.756-1.628 (pentet; H-4' and H-4''); 1.475-1.285 (complex multiplet; H-5' / H-5'' H-6' / H-6'' H-7' / H-7'' and H-9' / H-9'') 1.003-0.895 (complex multiplet; H-8' / H-8'' and H-10' / H-10'')

Peak integration ratio = 1.00: 1.77: 9.32: 6.70; ~2H: 4H: 19H: 13H = 38 protons

The presence of aromatic protons (two doublets of a doublet, dd) indicates 4 protons in which a pair (H-2/H-5) and (H-3/H-4) are in the same chemical environment (ortho-substituted benzene). The septet is most likely the result of H of CH_2 (H-3'/H-3'') germinal to $RCOO^-$ group, while, the pentet is indicative of an H of CH (H-4'/H-4'') bonded to two methylenes. The complex multiplet indicates several hydrogen of CH_2 groups (H-5'/H-5'', H-6'/H-6'', H-7'/H-7'' and H-9'/H-9'') in the same chemical environment, hence the overlapping signals. More multiplets at upfield indicate terminal protons of CH_3 attached to a CH_2 (H-8'/H-8'' and H-10'/H-10''). The peak integration ratio showed that the compound is made up of 38 protons.

^{13}C -NMR (ppm): 167.75 (carbonyl, C-1); 132.47 (aromatic, C-1 and C-6); 130.87 (aromatic, C-3 and C-4); 128.80 (aromatic, C-2 and C-5); 68.16 (C-5); 38.74 (C-6); 31.43 (C-7); 29.70 (C-8); 22.98 (C-9); 19.15 (C-10); 14.04 (C-11) and 10.95 (C-12)

Carbon-13 NMR revealed a peak at low field showing a de-shielded carbonyl group attached to an aromatic ring. A strong peak up field of the aromatic peaks indicates the presence of oxygen atom attached to a methylene group. The spectra revealed a total of 12 carbons atoms (although GC-MS spectrum revealed 24), an indication that the compound consisted of two identical parts, which are mirror images of each other¹⁸. The presence of a plane of symmetry in the molecule was confirmed by the small observed optical rotation.

DEPT-135 (ppm): no peaks (C-1, carbonyl and C-2, aryl); 130.88 and 128.80 (C-3 and C-4, methine of aromatic); 68.16 (C-5, methylene); 38.74 (C-6, methine); 30.37, 28.93, 23.75 and 22.98 (C-7, C-8, C-9 and C-10, methylene); 14.04 and 10.96 (C-11 and C-12, methyl).

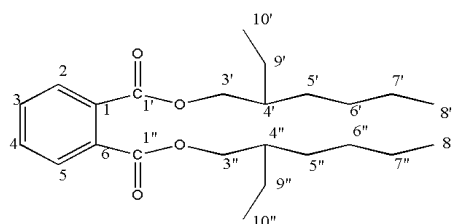
Distortion enhancement by polarization at pulse 135° revealed that the compound is made up of two quaternary carbons (disappeared in the spectrum), two methyl, three methine and five methylene carbons.

GC-MS (m/z): 391 [$C_{24}H_{38}O_4$] +1; 390 [$C_{24}H_{38}O_4$] + 1; 261 M- [$C_8H_{17}O$]; 168 [$C_8H_8O_4$]⁺; 167 [$C_8H_7O_4$]⁺; 149 [$C_8H_5O_3$]⁺; 113 [C_8H_{17}]⁺; 104 [C_7H_4O]⁺; 84 [C_6H_{12}]⁺; 71 [C_5H_{11}]⁺; 57 [C_4H_9]⁺; 41 [C_3H_5]⁺ and 27 [C_2H_3]⁺

The GC-MS fragmentation patterns revealed the compound to be a phthalate, with characteristic peaks at m/z 149, 167 and 168¹⁹.

Correlation of the obtained spectral data with those published in literature^{20, 21, 18, 22, 23, 24, 8} revealed the compound to be 1, 2-benzenedicarboxylic acid, dioctyl ester, also known as di-(2-ethylhexyl) phthalate (DEHP) or bis-(2-ethylhexyl) phthalate (BEHP) as shown in Figure 4. This bioactive compound was earlier isolated from the acetone-soluble portion of the ripe fruits of *N. latifolia*, but in greater quantity than that of the unripe fruits⁸. The presence of such a phthalate in a plant, not as a pollutant from solvents, nor plasticizers used during extraction and sample preparation could be ignored as the source of our isolate, because our experiment was repeated twice at different periods to ensure that the compound was a natural product and not an impurity.

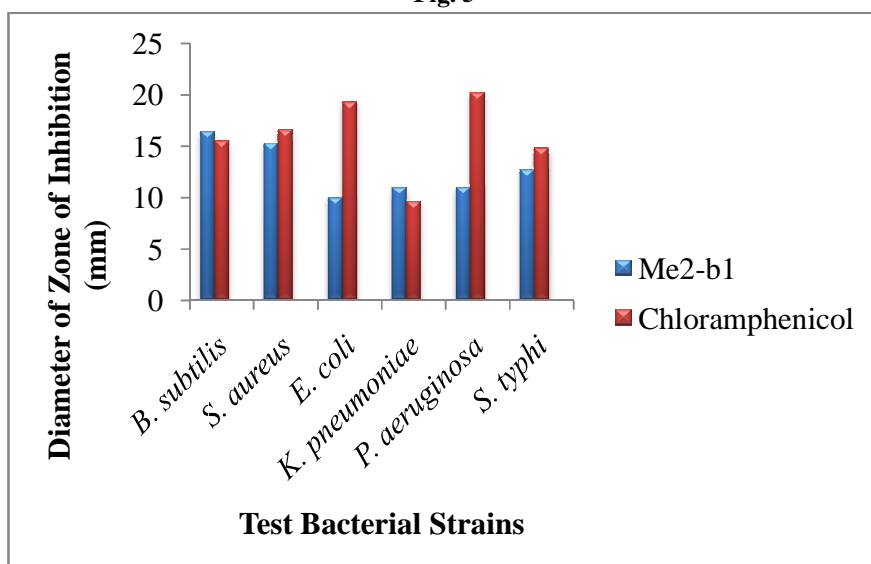
Fig. 4



1, 2-benzenedicarboxylic acid, dioctyl ester/Phthalic acid, bis (2-ethylhexyl) ester/ di-(2-ethylhexyl) phthalate/ bis-(2-ethylhexyl) phthalate.

Antibacterial study of the isolated compound, Me2-b1, showed that it displayed moderate inhibitory activity at 100 μ g/ml against *Gram* positive *B. subtilis* and *S. aureus*, an activity similar to that displayed by chloramphenicol at 1mg against these organisms (Figure 5). This supports the findings of other authors^{18, 22, 23, 24, 8}.

Fig. 5



Antibacterial activity of the isolated compound, Me2-b1 (100 μ g/ml) in comparison with chloramphenicol (1mg/ml) against test bacterial strains

CONCLUSION

The data obtained in the present work reports for the first time the isolation and characterization of 1, 2-benzenedicarboxylic acid, dioctyl ester from the unripe fruits of *N. latifolia*. The compound, like chloramphenicol could make a good candidate for the treatment of infections caused by *Gram* positive *Bacillus subtilis* and *Staphylococcus aureus*.

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