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Antibacterial Activity-Guided Isolation of Di-(2- Ethylhexyl) Phthalate from the Acetone-Soluble Portion of the Ripen Fruits of *Nauclea latifolia*

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Abstract
Traditionally, the fruits of *Nauclea latifolia* (Family Rubiaceae) are recommended for the treatment of diarrhea, dysentery and other bacterial infections. This therefore, prompted the phytochemical and *in-vitro* antibacterial assessment of the acetone-soluble portion of the plant. Crude extraction, fractionation, sub-fractionation, further purifications and various antibacterial testing gave rise to the isolation of di-(2-ethylhexyl) phthalate (DEHP) for the first time from the plant. The structure of the compound was elucidated using physical, chemical, literature search and spectroscopic identification (IR, UV, ^{1}H-NMR, ^{13}C-NMR, GC- MS and DEPT-135). Although, DEHP is regarded as an environmental pollutant, it displayed moderate inhibitory activity at 100µg/ml against Gram positive organisms, similar to that exhibited by tetracycline at 1mg/ml against the test organisms. This shows that DEHP, most likely may not be too useful in the treatment of diarrhea and dysentery caused by *Gram negative Escherichia coli, Pseudomonas aeruginosa and Salmonella typhi*, but may be useful in the treatment of diarrhea, dysentery and other bacterial infections caused by *Gram* positive *Bacillus subtilis* and *Staphylococcus aureus*. Though, the toxicity of DEHP still needs to be ascertained viz- a-viz its antibacterial effect.

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1. Introduction

1.1 Natural products

Natural plant products are rich sources of various bioactive compounds that exhibit interesting anti-microbial, anti- viral, anti-inflammatory activities etc, making natural products an important source for the discovery of new pharmaceuticals that are highly effective with low toxicity. They, therefore, constitute a practical endless source of novel substances that are able to enrich therapeutics (Portier et al., 1996).

1.2 Literature review

The plant, *Nauclea latifolia* Smth (Family: Rubiaceae) is a small tree of about 7m high, but could grow up to 35 m in closed forests. The 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 pulp (Iwu, 1993). The dried fruit is used traditionally in the treatment of dysentery, diarrhea and piles (Iwu et al., 1999) while its extract has been shown to be active against Human Immune Deficiency Virus (Hussein et al., 1999). Earlier work carried out on the fruits, 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 fruits (Fadipe et al., 2013). Non- medicinally, the plant is regarded as a source of food in Sudan and Northern Nigeria (Abdelmuti, 1991; Aiyela'agbe et al., 1996). The fruit is reportedly rich in vitamin C and this has made it a good source of fruit juice (Amoo and Lajide, 1999).

2. Objective of Research

The development of resistance in infectious micro-organisms to existing drugs has led to the continual search for, and the interest in natural plant products for use as medicines. This has therefore, prompted the use of bio-activity guided isolation and characterization of bioactive constituents from such plants. The plant, *Nauclea latifolia* had earlier been described as one of the plants with promising anti- infective activity/interesting biological activity (Iwu et al., 1999). This led the authors to investigate the antibacterial activity of the acetone-soluble portion of the ripe fruits of *N. latifolia* and attempt to isolate and characterize any antibacterial agent (s) from this portion. This hopefully could help provide more biologically active constituents which may be used to develop more safe and potent antibacterial life-saving drugs. A review of the literature reveals no such investigation has been reported. This work, therefore, presents the result of the antibacterial activity-guided isolation of a phthalic acid derivative from the acetone- soluble portion of ripe fruits of *N. latifolia* against some pathogenic bacteria in comparison with tetracycline, a reference drug.

3. Experimental

Work plan
i) Preparation of plant sample (4 weeks)
ii) Preparation of extract/portion/antiba cterial studies (4 weeks)
iii) Column chromatographic separation of portion/antibacterial studies (3 weeks)
iv) Purification /fractionat ion of active fractions (sub- fractions)/antibacteria l studies (8 weeks)
v) Isolation/characterizat ion of isolate (8 weeks, Ethiopia)
iv) Interpretation of results (4 weeks)

3.1 Collection of plant material

The ripe fruits of *N. latifolia* were collected from a farmland in Maikunkele area of Bosso Local Government area, Minna, Niger State, Nigeria, West Africa, in the month of October, 2010. The fruits were authenticated at the Herbarium section of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria, West Africa, and a voucher specimen was deposited.

3.2 Extraction procedures

Air-dried, pounded and sieved ripe 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.

3.3 Further extraction and Antibacterial study

Defatted crude methanol extract (457g) was suspended in 1L of distilled water and allowed to stand for 2h. The mixture was filtered to obtain 213g of water- insoluble portion (residue). The residue was air-dried and re-extracted exhaustively with 200 ml x 5 portions of acetone. The resulting filtrate was concentrated, dried, weighed and coded acetone-soluble portion of water-insoluble portion of defatted crude methanol extract of ripe fruits of *N. latifolia* (Mac). Crude 'Mac' was subjected to antibacterial screening in comparison with tetracycline. (Figure 3.1)

3.4 Fractionation of Mac

Forty grams of portion 'Mac' was fractionated using vacuum liquid chromatography (Pelletier et al., 1986). 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- methanol was used as the mobile phase. Similar fractions were pooled using TLC and combined similar fractions were subjected to antibacterial testing (Figure 3.2).

3.5 Further purification of fraction Mac-5

Eight grams of fraction Mac-5 was subjected to further purification by flash column chromatography using silica gel (mesh 230-400, 160g) and varying proportions of petroleum ether-chloroform as stationary and mobile phases respectively. Pooled sub-fractions were also subjected to antibacterial testing (Figure 3.3).

3.6 Further purification of sub-fraction Mac-5a

Purification of sub- fraction Mac-5a (600mg) using flash chromatography (silica gel mesh 230-400, 30g) and increasing polarity of hexane: chloroform afforded a single spot (coded Mac-5a1) from a mixture of hexane: $CHCl^3$ (1:9).

3.7 Characterization of Mac-5a1

Chromatograms were viewed under UV (254 and 366nm), I_2 and sprayed with anisaldehyde-sulphuric acid.

Melting point was uncorrected. Rotation (Rudolph Autopol IV automatic polarimeter), FTIR (Spectrolab MB3000) and UV spectra (T60 UV-Visible spectrophotometer) was taken in $CHCl_3$; ^{1}H-NMR (Table 3.1) ^{13}C-NMR (Table 3.2) and DEPT-135 (Table 3.3) spectra were taken in $CDCl_3$ on Bruker ACQ 400 Avance spectrometer operating at 400MHz. GC-MS was recorded using GCMS-QP 2010 plus Shimadzu (Table 3.4).

3.8 Antibacterial testing

3.8.1 Bacterial cultures

The antibacterial activity of crude portion/fractions/sub- fractions/isolate was 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.

3.8.2 Bacterial susceptibility testing

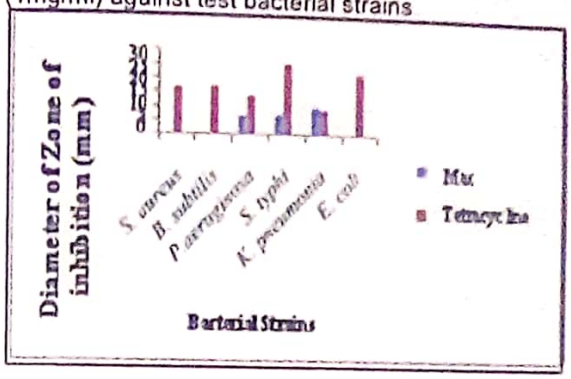
The agar-well diffusion method was employed (Perez et al., 1990; Dall'Agnol et al., 2003). Standardized inoculums containing 10^6 cfu/ml 0.5 ml McFarland standards were evenly streaked onto the surface of sterile agar plates for each organism. 8mm wells were bored into the solidified agar using sterile cork borer at equidistant. 100µl of reconstituted Mac (100 mg/ml), Mac-5 (20mg/ml), Mac-5a to Mac-5c (20 mg/ml), Mac-5a1 (100µg/ml), positive control (Tetracycline, 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 ≥ 14mm (Mothana and Linderquist, 2005).

4. Results and Discussion

4.1 Antibacterial activity of crude portion (Mac)

The crude acetone-soluble portion of the ripe fruits of *N. latifolia* (Mac) was practically inactive against all test organisms when compared to tetracycline (Figure 3.1).

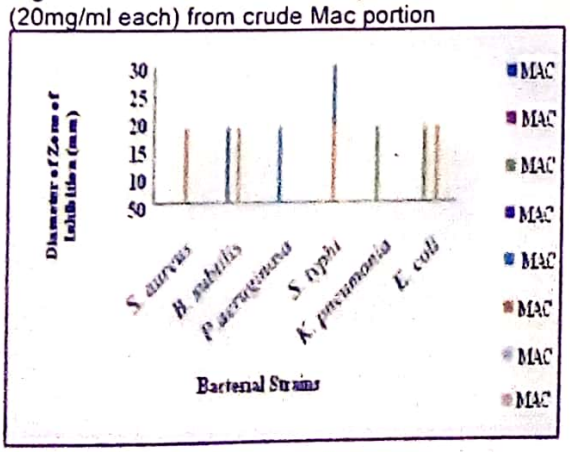
Figure 3.1: Antibacterial activity of 'Mac' (100mg/ml) in comparison with tetracycline (1mg/ml) against test bacterial strains



4.2 Antibacterial activity of fractions from portion 'Mac'

Fractionation of the 'inactive Mac' by vacuum liquid chromatography gave rise to 8 fractions (Figure 3.2) of which fraction Mac-5 gave a significant inhibitory activity at 20mg/ml against *Gram* negative *S. typhi*, *E. Coli* and *Gram* positive *B. subtilis*. Sometimes, the amount of active components in crude extracts/portions from medicinal plants may be small or too diluted, so that when fractionated, these components become concentrated and therefore exhibit better activity (Ndip et al., 2009). Fractionation of a crude plant extract has reportedly improved the antimicrobial potency of some medicinal plants (Sharifi and Hazell, 2009; Nazemi et al., 2010).

Figure 3.2: Antibacterial activity of the 8 fractions (20mg/ml each) from crude Mac portion

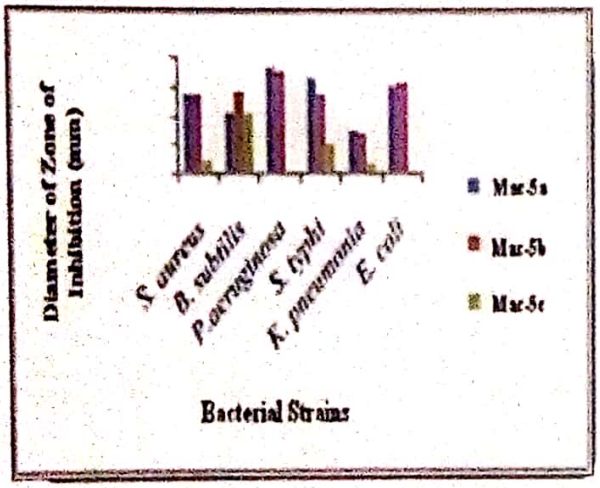


4.3 Antibacterial activity of sub- fractions from fraction 'Mac-5'

Antibacterial assay of the three sub-fractions obtained from the re-purification of fraction Mac-5 by column chromatography (Figure 3.3) revealed that sub- fractions Mac-5a and Mac-

5b displayed significant inhibitory activities against the Gram- negative than the Gram positive strains, an activity much lower than that displayed by fraction Mac-5 against *S. typhi*. This might be as a result of may be these sub-fractions contain 'inactive substances' which probably antagonized/ reduced the antibacterial action of one another (Ebi and Ofoefule, 1997) or their activity could be enhanced at higher concentrations, or better still, the stronger activity of Mac-5 may

Figure 3.3: Antibacterial activity of the 3 major sub-fractions (20mg/ml each) against test bacterial strains



Both sub-fractions Mac-5a and Mac-5b gave similar inhibitory zones against all the test organisms, suggesting that probably both sub-fractions possess common/similar bioactives that is/are responsible for the displayed activity against the test organisms. The biological activity of plant extracts, probably reflect contributions from a number of constituents (Ndip et al., 2009).

4.4 Characterization of compound Mac-5a1

4.4.1 Physical characterization

A golden yellow viscous oil (32.6mg), slight odor, soluble in chloroform, acetone, methanol and insoluble in water. Spot (petroleum ether: EtOAc 9: 1) was uv active (red), R_f (0.40, red when sprayed with anisaldehyde- sulphuric acid; golden brown be due to combined with I2 crystals; no color with effects/synergism of both sub- fractions, Mac-5a and Mac-5b. (Fadipe et al., 2006; Nazemi et al., 2010). sunlight). Molecular formula $C_{24}H_{38}O_4$; Molecular weight: 390.56; Melting point:-54° C, [α]22 D (589nm): 0.588, $[\alpha]^{22}$ D (633nm): 1.176.

3.4.2 Spectral characterization

UV (λ max): 275nm (C-O, carbonyl) IR (v max): 2900-2850 cm^{-1} (C- H, aliphatic), 1739 cm^{-1} (C-O, carbonyl), 1578 cm-1(aromatic ring), 1200cm^{-1} (C-O, acetate).

Table 3.1: ^{1}H-NMR data of Mac- 5a1

Position	^{1}H (ppm)	Assignment
H-2 & H-5	7.740- .709 (dd)	Indicates presence of aromatic protons (Ar-H)
H-3 & H-4	7.534-7.557 (dd)	..
	7.256 (singlet)	Indicates solvent, $CDCl_3$
H-3'/H-3"	4.195-4.280 (septet)	Indicates presence of sp^3 protons attached to oxygenated sp^3 carbon ($O-CH_2$-)
H-4'/H-4"	1.628-1.756 (pentet)	Indicates presence of CH-attached to CH_2- groups
H-5'/H-5" H-6'/H-6" H-7'/H-7" H-9'/H-9"	1.475-1.285 (complex multiplet)	Overlapping signals of several CH_2- groups
H-8'/H-8" H-10'/H-10"	0.895-1.003 (multiplet)	Overlapping signals of CH_3 - groups attached to CH_2- groups

Proton NMR revealed the presence of four aromatic protons in which a pair (H at position 2 and 5) and (H at 3 and 4) are in the same chemical environment (ortho-substituted benzene). The septet at ~δ 4.28 is most likely the result of a CH_2 (position 3'/3") germinal to RCOO- group, while, a pentent at ~δ 1.62 is indicative of a CH (position 4'/4") bonded to two methylenes. A complex multiplet at ~δ 1.2 to ~δ1.4 indicates several CH_2 groups (positions 5'/5", 6'/6", 7'/7" and 9'/9") in the same chemical environment, hence the

overlapping signals. Peaks at ~δ 0.89 to 1.00 indicate a terminal CH_3 attached to a CH_2 (positions 8'/8" and 10'/10").

The peak area integration ratio: 1.00: 1.07: 2.11: 1.11: 7.11: 0.71: 6.42= ~2H: 2H: 4H: 2H: 14H: 1H: 13H = ~38 protons, showing that the compound is made up of 38 protons.

Table 3.2: ^{13}C-NMR data of Mac-5a1

Position	^{13}C(ppm)	Assignment
C-1	167.75	Weak signal, indicates -CO
C-2	132.47	Aromatic C atom (C-1& C-6)
C-3	130.87	,, (C-3 & C-4)
C-4	128.80	,, (C-2 & C-5)
C-5	68.16	Presence of -O-CH_2
C-6	38.74	Presence of -CH
C-7	30.37	Presence of -CH_2 -C
C-8	28.93	,,
C-9	23.75	,,
C-10	22.98	,,
C-11	14.04	Presence of a -CH3
C-12	10.95	,,

Carbon 13 NMR revealed the presence of only 12 carbon atoms (although GC-MS spectrum revealed 24; Table 3.4), an indication that the compound consisted of two identical parts, which are mirror images of each other (Alim Al-Bari et al., 2006). The presence of a plane of symmetry in the molecule was confirmed by the small observed optical rotation (αD) at two different wavelengths, an indication that that the molecule is only slightly chiral/almost achiral/ a meso compound (Jones, 1997), having C-4'/C-4" as the only chiral centre.

Table 3.3: DEPT-135 data of Mac-5a1

Position	DEPT-135 (ppm)	Assignment
C-1	167.75	Presence of quaternary C (–CO)
C-2	132.47	,, (Ar-)
C-3	130.87	Presence of a methine (-CH of aromatic)
C-4	128.80	,,
C-5	68.16	Presence of a methylene (-CH_2 attached to -OR)
C-6	38.74	Presence of methine (-CH-)
C-7	30.37	Presence of methylene (-CH_2 -)
C-8	28.93	,,
C-9	23.75	,,
C-10	22.98	,,
C-11	14.04	Presence of methyl (-CH_3)
C-12	10.95	,,

Among the 12 carbon resonances obtained, DEPT-135 revealed that two were quaternary (disappeared in the spectrum) while, two, three and five were methyl, methine and methylene carbon atoms.

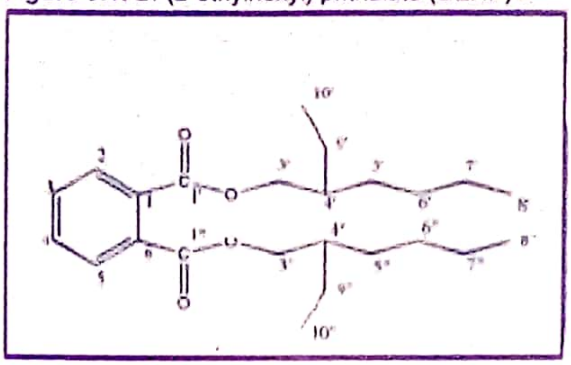
Table 3.4: GC-MS of Mac-5a1

Fragment (M)	m/z of fragment ion	Assignment
$[C_{24}H_{38}O_4]$+ 1	391	Molecular ion peak+1 [M + H]+
$[C_{24}H_{38}O_4]$	390	Molecular ion peak [M]
M-$[C_8H_{17}O]$	261	Elimination of -OR
$[C_8H_6O_4]$+	168	Rearrangement of two H atoms with elimination of an allylic radical
+ $[C_8H_7O_4]$	167	McLafferty rearrangement
+ $[C_8H_5O_3]$	149	Cleavage of two esters with shift of two H atoms, followed by elimination of H_2O
$[C_8H_{17}]$+	113	Retention of the positive + charge by the alkyl group (R)
$[C_7H_4O]$+	104	Elimination of Ar-CO+
+ $[C_6H_{12}]$	84	Elimination of hexyl cation
+ $[C_5H_{11}]$	71	Elimination of pentyl cation
+ $[C_4H_9]$	57	Elimination of butyl cation (base peak ion)
+ $[C_3H_5]$	41	Elimination of propyl cation
+ $[C_2H_3]$	27	Elimination of ethyl cation

The GC-MS fragmentation patterns revealed the compound to be a phthalate, with characteristic peaks at m/z 149, 167 and 168 (Silverstein et al., 1991).

By comparison of the obtained spectral data with those published in literature (Amade et al., 1994; Rao et al., 2000; Alim Al-Bari et al., 2006; Habib and Karim, 2009; Lyutskanova et al., 2009; El-Sayed, 2012), the compound was identified as Di- (2-ethylhexyl) phthalate (DEHP) or Bis-(2-ethylhexyl) phthalate (BEHP).

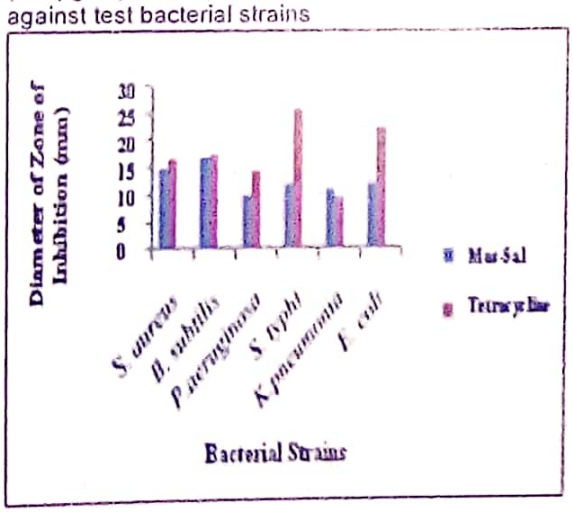
Figure 3.4: Di-(2-ethylhexyl) phthalate (DEHP)



3.5 Antibacterial activity of compound Mac-5a1

DEHP displayed moderate inhibitory activity at 100µg/ml against *Gram* positive *B. subtilis* and *S. aureus*, an activity similar to that displayed by tetracycline at 1 mg/ml against these organisms (Figure 3.5). This suggests that the compound might be useful in the treatment of infections caused by these organisms. This supports the findings of other authors (Alim Al-Bari et al., 2006; Habib and Karim, 2009; Lyutskanova et al., 2009; El-Sayed, 2012).

Figure 3.5: Antibacterial activity of Mac-5a1 (100µg/ml) in comparison with tetracycline (1mg/ml) against test bacterial strains



Limitations

i. Inadequate test pathogens
ii. Non-availability of required equipments within my reach.

Recommendations

i. There should be further isolation/antibacterial studies of other active principles present in both the ripe and unripe fruits for comparative purpose.
ii. The antimicrobial potentials of DEHP against a wider range of Mac-5a1 Tetracycline bacteria, fungi and virus should be studied.
iii. Toxicological studies to determine the safety of DEHP should be carried out.
iv. The mode of action of DEHP against the bacteria should be studied.
v. It is pertinent to study the effect of DEHP on methicillin resistance coagulase negative Staphylococcus and methicillin resistant or multi-drug resistant (MDR) *S. aureus*.

Conclusion the data obtained in the present work reports for the first time the isolation and characterization of di- (2-ethylhexyl) Phthalate (DEHP) from the ripe fruits of *N. latifolia*. It was found that DEHP, like tetracycline could make a good candidate for the treatment of infections caused by *Gram* positive Bacillus subtilis and Staphylococcus aureus.

Conclusion

The data obtained in the present work reports for the first time the isolation and characterization of di- (2-ethylhexyl) Phthalate (DEHP) from the ripe fruits of N. latifolia. It was found that DEHP, like tetracycline could make a good candidate for the treatment of infections caused by *Gram* positive *Bacillus subtilis* and *Staphylococcus* aureus.

Author's Contribution and Competing Interest

DEHP, like many phthalate derivatives, are ubiquitous chemicals that are considered as 'environmental contaminants or pollutants'. They have been detected in soil sediments, terrestrial and marine waters and in living organisms (Namikoshi et al., 2006). Although, they are sometimes disregarded in considerations of risk (Rhind et al., 2005) because on the basis of chemical structure, it is considered that they are readily degraded in the environment (Smith, 1995). Therefore, presence of such a phthalate in a plant might not be a pollutant from environment, solvents, nor plasticizers used during extraction and sample preparation. Our experiment was repeated twice at different periods to ensure that the compound was a natural product and not an impurity. Such phthalates, like DEHP, could be useful as an antimicrobial agent.

Acknowledgement

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