

## Original Research Article

### Isolation and Antimicrobial Activity of ~~Bioactive~~ Methylated Flavanone from Methanol Stem-Bark extract of *Nauclea diderichii* (~~Rubiaceae~~)

#### ABSTRSCT

The chromatographic investigation of the ethyl-acetate soluble fraction of methanol stem bark extract of *Nauclea diderichii* using a combination of silica and sephadex LH-20 led to the isolation of a methylated flavanone. The structure of this new compound was determined using both 1 and 2D nuclear magnetic resonance (NMR). The antimicrobial activities of the crude fractions were also investigated using the agar diffusion and broth dilution techniques. Clinical isolates obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria were used for the studies. The crude fraction showed activity against *Staphylococcus aureus*, *Streptococcus pyrogenes*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Samonella typhi*, but were tested doses below detectable limits (BDL) in methicillin resistant *S. aureus*, *Bacillus subtilis*, *Corynobacterium ulcerans*, *Escherichia coli* and the fungus *Candida albicans* (needs more clarification). The low concentration for minimum inhibitory concentration (MIC, 1.25 µg/mL) and minimum bactericidal concentration (MBC, 2.25 µg/mL), while minimum fungicidal concentration (MFC, 5.00 µg/mL) suggesting that the compound has a good antimicrobial activity against the susceptible organisms and validates the ethno-medical use of the *N. diderichii* in the treatment of various bacterial infections, including the opportunistic HIV (needs more clarification, infact HIV is not a bacterium).

**Key words:** *Nauclea diderichii*, antimicrobial, nuclear magnetic resonance (NMR). Microbes, secondary metabolites (needs to write properly with reasonable keys).

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## 26 INTRODUCTION

27 Bioactive plant secondary metabolites have been found to be active against (please use only one) a wide  
28 variety of micro-organisms and have shown great promise in the treatment of many intractable diseases  
29 like the opportunistic infectious diseases and even the recent Ebola virus. Most of the plant secondary  
30 metabolites have been investigated for their chemical components and some of the isolated compounds  
31 have been shown to possess diverse and interesting biological activities (Gupta, 1994; Eja *et al.*, 2007)  
32 (please insert new references).

33 Malaria, diarrhoea and dysentery are health challenges (Nwankwo *et al.*, 2005; Emokpae *et al.*, 2006) and  
34 isare believed that *Nauclea diderichii* possesses medicinal properties effective in their management since  
35 decoctions of this plant are used for their cure in Mubi North and South LGAs of Adamawa State North-  
36 Eastern Nigeria.

37 *Nauclea diderichii* (De Wild & Dur) Meril (Rubiaceae). Its commercial name is 'bilinga' and common  
38 name is 'African peach', is reputed (known!) for its numerous medicinal uses as a tonic and fever  
39 medicine (antipyretic!), chewing stick, toothaches; dental caries, septic mouth, malaria, diarrhoea and  
40 dysentery cure (Lamidi, 1995).

41 Most European countries similarly witness a seemingly unimpeded increase of antimicrobial resistance in  
42 the major Gram-negative pathogens which could unavoidably lead to loss of therapeutic treatment options  
43 (European Centre for Disease Prevention and Control (ECDC), 2012).

44 Natural products have been the most significant source of drugs and drug leads in history (Cragg and  
45 Newmann, 2005). The emergence of multidrug resistance in human and animal pathogenic bacteria as  
46 well as undesirable side-effects of certain antibiotics has triggered immense interest in the search for new  
47 antimicrobial / antifungal / antiviral drugs of plant origin (Ahmed and Beg, 2001). In theory, bacteria will  
48 continue to develop resistance once exposed to any antimicrobial agents, thereby imposing the need for a

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49 permanent (continuous!) search, develop and advancement of new drugs (Silver and Bostian, 1993) and  
50 higher plants represent a potential source of novel antibiotic prototypes (Meurer–Grimes *et al.*, 1996).

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51 In Mubi LGAs, North Eastern Nigeria, the stem bark of *bilinga* are used (oral decoction), for the  
52 treatment of malaria and dysentery /diarrhoea both in children and adults (per community).

53 In our search for bio-active compounds from plants, we hereby report the isolation and antimicrobial  
54 effect of a new methylated flavanone from the ethyl acetate soluble fraction of the methanol extract of the  
55 stem-bark of ~~*Nauclea*~~ *N. diderichii*, thus viewing the plant as a potential source for phytomedicine  
56 development.

## 57 MATERIALS AND METHODS

58 **Identification, collection and preparation of plant:** Fresh samples of the stem bark of the *N.*  
59 *diderichii* ~~African peach~~ were obtained from Michika and Bali in Adamawa and Taraba States,  
60 respectively, in September, 2011 and were identified in the Biological Sciences Department of Adamawa  
61 State University Mubi. The FHI number is 0194 and a specimen of the plant was deposited in the  
62 herbarium. The sample (1.4kg) was air dried in the laboratory before pounding to a fine powder using  
63 pestle and mortar to about 70 mesh sizes and then stored in a dry sterile bottles.

64 **Extraction:** 450g of the powdered stem bark was accurately weighed and percolated with 10L of *distilled*  
65 *methanol* (*is it!*) for 72 *hours*. After which there was decantation, filtration, and concentration using a  
66 rotary evaporator (R110) at 35°C to obtain *the* methanol soluble fractions, (F<sub>m</sub>01), labelled, F<sub>m</sub>0R. [30.5g]  
67 and this was kept in the refrigerator for further separation. It was later macerated with different solvents  
68 of increasing polarities starting with petroleum ether, chloroform, ethyl-acetate and methanol to have their  
69 corresponding fractions.

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70 **Thin layer chromatography (TLC):** The ethyl-acetate soluble portion of the methanol extract of *N.*  
71 *diderichii*, was subjected to TLC using pre-coated aluminum plates. The solvent systems used were ethyl-

72 acetate: methanol (9:1). The spots on TLC were visualized under ultraviolet (UV), spraying with 5%  
73 sulphuric acid in methanol followed by heating at a temperature of 110°C for 5 minutes.

#### 74 Chromatographic separation

75 **Column chromatography, silica gel (60 -80):** Ethyl acetate soluble fraction (2.5-g) [\(please ensure the](#)  
76 [appropriate formatting throughout the text: as it is necessary to maintain a space between value and unit](#)  
77 [or not!\)](#) was mounted on a glass column (75 × 3.5 cm). The column was eluted continuously using  
78 petroleum ether, ethyl acetate and methanol mixture by gradient elution technique; 20 mL each was  
79 collected as eluates and the progress of elution was monitored using TLC. A total of 15 fractions (ND-01-  
80 ND-15) from 200 collections were made; similar fractions were combined and further purified by  
81 Sephadex LH-20 gel filtration column chromatography. In both cases, the progress of separation was  
82 monitored using TLC.

83 **Gel filtration:** Further purification of ND-06 was undertaken using sephadex LH-20 eluted with ethyl  
84 acetate. The progress of separation was monitored using TLC. Repeated gel filtration led to the isolation  
85 of a compound coded ND-06.

86 **Structure determination:** Spectral structure determination was conducted at the School of Chemistry,  
87 University of Kwazulu-Natal, South Africa using UV light at 356 nm, infra-red (IR) was recorded at 3239.9  
88 cm<sup>-1</sup>, NMR was run with 400 MG-Htz Bruker NMR machine [\(check the front\)](#) and data confirmed using  
89 MestReNova<sup>(R)</sup>.

#### 90 Antimicrobial assay:

91 **Microorganisms/strains:** The microorganisms tested included *Staphylococcus aureus*, methicillin  
92 resistant *Staphylococcus S. aureus*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Corynebacterium ulcerans*,  
93 *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella typhi* and *Candida*  
94 *albicans* [\(if it's possible please insert the strain number\)](#). All the organisms were clinical isolates obtained

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95 from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria,  
96 Nigeria.

97 **Reference drug:** Sparfloxacin 5 mcg/disc made by Himedia Laboratories Pvt. Ltd. Mumbai, India was  
98 used as the reference drug.

99 **Susceptibility studies:** Preliminary antimicrobial activity of the compound was carried out using stock  
100 concentration of 20 µg/mL. The microorganisms were maintained on agar slant. The inocula were  
101 prepared by inoculating the test organisms in nutrient broth and incubating them for 24 h at 37°C for  
102 the bacteria, while for *C. albicans*, Sabouraud Dextrose broth was used and was incubated for 48 h. After

103 incubation, the broth cultures were diluted to 1:1000 for the Gram-positive bacteria and 1:5000 for the  
104 Gram-negative bacteria. One milliliter of the diluted cultures was inoculated into a sterile molten nutrient  
105 agar at 45°C and poured into sterile petri-dish. Similarly, 1 mL of the diluted fungal suspension was  
106 poured into sterile Sabouraud dextrose agar plates and the excess sucked up with Pasteur pipette. These  
107 were swirled gently and allowed to solidify. Wells were bored into the solidified inoculated nutrient agar

108 plates using cork borer of 6 mm diameter. The wells were filled with 0.1 mL of the compound.  
109 Sparfloxacin standard disc was also placed on the agar plate. A one hour ~~1-h~~ was allowed for the  
110 compound to diffuse into the agar after which the plates were incubated overnight at 37 and 25°C for  
111 bacteria and fungi, respectively. At the end of incubation period, diameter of inhibition zone was  
112 measured using transparent ruler and recorded. The compound and standard antibiotic were tested in  
113 duplicate and mean zones of inhibition were computed (please insert a reference; if not, before that you  
114 can denote as modification method of-----).

#### 115 **Minimum inhibitory concentration (MIC)**

116 MIC was determined using broth dilution method (Volleková *et al.*, 2001). Two fold (half-fold!) serial  
117 dilutions of the compound were made to obtain concentrations of 5.00, 2.50, 1.25, 0.625 and 0.312  
118 µg/mL. 0.2 mL suspension of standard inoculum of each organism was inoculated to the different  
119 concentrations of the compound. The test tubes were then incubated at 37°C for 24 h after which they

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120 | were observed for the growth. Inhibition of growth was indicated by a clear solution. The MIC was  
121 | defined as the lowest concentration of the compound inhibiting the visible growth of each microorganism.

122 | **Minimum Bactericidal Concentration (MBC) / Minimum Fungicidal Concentration** (please check:  
123 | if each word should be capitalized or not)

124 | The contents of the MIC tubes and the preceding tubes in the serial dilution were sub-cultured into the  
125 | appropriately labeled nutrient agar plates by dipping a sterile wire loop into each test tube and streaking  
126 | the surface of the labeled nutrient agar plates. The plates were then incubated at 37°C for 24 h after which  
127 | they were observed for colony growth. The lowest concentration of the sub-culture with no growth was  
128 | considered as ~~minimum bactericidal concentration~~MBC / ~~MFC~~minimum fungicidal concentration  
129 | (Volleková *et al.*, 2001).

## 130 | RESULTS AND DISCUSSION

131 | **EXTRACTION:** The crude methanol extract (850 g) of *N. diderichii* stem bark after extraction, was  
132 | macerated with petroleum ether, chloroform and ethyl acetate to give the following residues and  
133 | weights (please check the front).

134 | **Table 1:** Extractive values of macerated methanol extract of *N. diderichii* stem bark (please check the  
135 | front)

| Extract       | Colour      | Weight (g) | Recovery yield (%) |
|---------------|-------------|------------|--------------------|
| Pet. Ether    | Light brown | 3.00       | 10.52              |
| Chloroform    | Pale yellow | 6.63       | 17.80              |
| Ethyl-acetate | Green       | 4.8        | 15,65              |
| Methanol      | Brown       | 24.4       | 28.70              |

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## 137 SPECTRAL DATA

138 **Ultraviolet (UV):** In ND-06, the UV absorption band using a 356 nm UV spectral lamp, giving a white  
139 spot is indicative of weak auxochromes that are pH dependent like hydroxyl group (OH). The C=C  
140 stretching frequency of the cyclic molecule could be seen at 1604cm<sup>-1</sup>.

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141 **Infrared (IR):** The IR spectra of the isolated compound indicated prominent absorption frequencies  
142 characteristic of certain functional groups: The band of medium intensity at 3239.9 cm<sup>-1</sup> suggests the  
143 presence of OH hydroxyl group(s). The broad band of very strong intensities at 2950 and 2852.76 cm<sup>-1</sup>  
144 are characteristic of C-H stretching of aliphatic groups e.g. CH<sub>3</sub>, CH<sub>2</sub>, and CH etc. Overtones and  
145 combination tones of lower and weak frequency bands are displayed at 1530.8 cm<sup>-1</sup>. Their appearance  
146 and position in a spectrum can be gainfully used for identification of some structural features. The C-H  
147 bending vibrations of the CH<sub>3</sub> and CH<sub>2</sub> groups are observed at 1429.6 and 1359.0 cm<sup>-1</sup>.

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## 148 Nuclear Magnetic Resonance (NMR)

149 **Proton (<sup>1</sup>H):** The chemical shift of any proton is determined by its chemical environment: The cluster of  
150 methyl and methylene protons as observed in the spectra suggests the flavanonal nature of the  
151 compounds. DEPT, 90, 45 and 135, provides a clue to the various methyls, methylenes, methines and  
152 quaternary carbons. The hydroxyl proton is again confirmed at 4.0969 ppm in the proton NMR spectral.

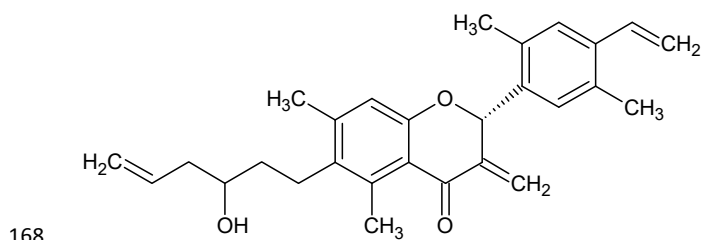
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153 **Carbon (<sup>13</sup>C):** Compound NDK12: Spectra data of <sup>1</sup>H, <sup>13</sup>C, HSQC, HMBC, COSY, DEPT, NOSEY and  
154 LC-MS spectroscopy confirm the structure to be a methylated flavanone (Figure 1), and this was  
155 confirmed and further justified by <sup>13</sup>C NMR data simulated by MestReNova<sup>(R)</sup> <sup>13</sup>C were within ± 03  
156 ppm (Reynolds et al., 1986; Tinto et al., 1992; Jain and Bari, 2010).

157 The NMR spectrum (Figure1) indicated thirteen quaternary, three methine, seven methylene, and five  
158 methyl carbons and there are other side groups attached on the flavanone skeleton. The hydroxyl moiety  
159 is further confirmed by the proton nuclear magnetic resonance <sup>1</sup>H-NMR by the band at 4.7350. The

appearances and positions of other combination peaks /bands in the spectrum have been gainfully used for  
identification of some structural features.

**Mass Spectra of ND-06:** Mass spectrum of ND-06: The mass spectrum of peak 0.11 minutes from the Gas Chromatogram showed the parent ion  $[M]^+$  peak at M/Z 419.1 which corresponds to the molecular formula  $C_{28}H_{32}O_3$  showing characteristics of mass spectrum for a methylated flavanone and on the bases of the (GC-MS and the inbuilt main library (C:/Database/NIST05.LIB search and confirmation of the compound(s) (Figure1) was confirmed as being present in the plant.



169 **Figure 1: Suspected structure (A Methylated flavanone)** (front; as the authors are giving mean  $\pm$  SEM  
170 data then there must be fraction mean? Please check the data calculation.)

**Table 2:** Zone of inhibitions for Petroleum ether, Chloroform, ethyl acetate and methanol extracts of the stem bark of *N. diderichii* on fourteen micro-organisms. (front)

| Micro-organisms        | Zones of inhibition (mm) |           |            |           |           |           |           |
|------------------------|--------------------------|-----------|------------|-----------|-----------|-----------|-----------|
|                        | Pet. Ether               | Chloro.   | Ethyl Act. | Methanol  | Sparflo   | Flocon    | DMSO      |
| Methicillin R.S.A      | 15 ± 0.11                | 25 ± 0.48 | 21 ± 0.63  | 21 ± 0.68 | 33 ± 0.68 | 35 ± 0.38 | 32 ± 0.67 |
| Staphylococcus aureus  | 15 ± 0.11                | 27 ± 0.76 | 21 ± 0.63  | 22 ± 0.46 | 35 ± 0.38 | 35 ± 0.38 | 32 ± 0.67 |
| Streptococcus pyogenes | 15 ± 0.11                | 27 ± 0.76 | 21 ± 0.63  | 22 ± 0.46 | 35 ± 0.38 | 35 ± 0.38 | 32 ± 0.67 |



176 **Table 3:** Minimum Inhibitory Concentration (MIC) of Petroleum ether, Chloroform, ethyl acetate and methanol extracts of the stem bark of *N.*  
 177 *diderichii* on fourteen micro-organisms (front: however, is it necessary to show all data which didn't have any MIC?)

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| Micro-organisms                 | Concentration (mg/mL or µg/mL?) |      |      |       |       |            |      |      |       |       |          |      |      |       |       |               |      |      |       |       |
|---------------------------------|---------------------------------|------|------|-------|-------|------------|------|------|-------|-------|----------|------|------|-------|-------|---------------|------|------|-------|-------|
|                                 | Petroleum Ether                 |      |      |       |       | Chloroform |      |      |       |       | Methanol |      |      |       |       | Ethyl acetate |      |      |       |       |
|                                 | 5.00                            | 2.50 | 1.25 | 0.625 | 0.312 | 5.00       | 2.50 | 1.25 | 0.625 | 0.312 | 5.00     | 2.50 | 1.25 | 0.625 | 0.312 | 5.00          | 2.50 | 1.25 | 0.625 | 0.312 |
| Methicillin R.S. aureus         | -                               | -    | -    | -     | -     | -          | -    | 1.25 | +     | ++    | -        | -    | 1.25 | +     | ++    | -             | -    | -    | -     | -     |
| <i>Staphylococcus aureus</i>    | 5.00                            | +    | +    | ++    | +++   | -          | -    | 1.25 | ++    | +++   | -        | -    | 1.25 | +     | ++    | -             | 2.50 | +    | ++    | ++    |
| <i>Streptococcus pyogenes</i>   | -                               | -    | -    | -     | -     | -          | -    | -    | -     | -     | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |
| <i>Bacillus subtilis</i>        | 5.00                            | +    | +    | ++    | +++   | -          | -    | 1.25 | +     | ++    | -        | 2.25 | -    | +     | ++    | -             | -    | 1.25 | +     | ++    |
| <i>Corynebacterium ulcerans</i> | -                               | -    | -    | -     | -     | -          | -    | 1.25 | +     | ++    | -        | -    | 1.25 | +     | ++    | -             | -    | 1.25 | +     | ++    |
| <i>Escherichia coli</i>         | -                               | 2.25 | +    | ++    | +++   | -          | -    | 1.25 | +     | ++    | -        | -    | 1.25 | +     | ++    | -             | -    | 1.25 | +     | ++    |
| <i>P.roteus mirabilis</i>       | -                               | -    | -    | -     | -     | -          | -    | 1.25 | +     | ++    | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |
| <i>P.roteus vulgaris</i>        | -                               | -    | -    | -     | -     | -          | -    | -    | -     | -     | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |
| <i>Pseudomonas aeruginosa</i>   | -                               | -    | -    | -     | -     | -          | -    | -    | -     | -     | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |
| <i>Salmonella typhi</i>         | -                               | -    | 1.25 | +     | ++    | -          | -    | 1.25 | +     | ++    | -        | -    | 1.25 | +     | ++    | -             | -    | 1.25 | +     | ++    |
| <i>Klebsella pneumoniae</i>     | -                               | 2.25 | +    | ++    | +++   | -          | -    | 1.25 | +     | ++    | -        | -    | 1.25 | +     | ++    | -             | -    | 1.25 | +     | ++    |
| <i>Candida albicans</i>         | -                               | -    | -    | -     | -     | -          | -    | 1.25 | -     | -     | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |
| <i>Candida stellatoidea</i>     | -                               | -    | -    | -     | -     | -          | -    | -    | -     | -     | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |
| <i>Candida tropicalis</i>       | -                               | -    | -    | -     | -     | -          | -    | 1.25 | +     | ++    | -        | -    | 1.25 | +     | ++    | -             | -    | 1.25 | +     | ++    |

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178 Key: - = no turbidity, (No growth), + = turbidity (Light growth), ++ = (Moderate growth), +++ = (High growth) MIC (Values Given) = Minimum  
 179 inhibitory concentration, Sparflo = Sparfloxacin (Bacteria Control): 10µg/mL, Flucona = Fluconazole (Fungal control): 20µg/mL

180 Please similarly follow the Table 4

181 **Table 4:** Minimum Bactericidal Concentration (MBC)/ Minimum Fungicidal Concentration (MFC) of Petroleum ether, Chloroform, ethyl acetate  
 182 and methanol extracts of the stem bark of *N. diderichii* on some fourteen micro-organisms

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| Micro-organisms                                 | Concentration (mg/mL) |      |      |       |       |            |      |      |       |       |          |      |      |       |       |               |      |      |       |       |
|-------------------------------------------------|-----------------------|------|------|-------|-------|------------|------|------|-------|-------|----------|------|------|-------|-------|---------------|------|------|-------|-------|
|                                                 | Pet. Ether            |      |      |       |       | Chloroform |      |      |       |       | Methanol |      |      |       |       | Ethyl acetate |      |      |       |       |
|                                                 | 5.00                  | 2.50 | 1.25 | 0.625 | 0.312 | 5.00       | 2.50 | 1.25 | 0.625 | 0.312 | 5.00     | 2.50 | 1.25 | 0.625 | 0.312 | 5.00          | 2.50 | 1.25 | 0.625 | 0.312 |
| Methicillin R.S.<br>aureus<br>Methicillin R.S.A | -                     | -    | -    | -     | -     | -          | -    | MIC  | +     | ++    | MIC      | +    | ++   | +++   | +++   | -             | -    | -    | -     | -     |
| <i>S.taphylococcus aureus</i>                   | MIC                   | +    | ++   | +++   | ++++  | -          | MIC  | +    | ++    | +++   | MIC      | +    | ++   | +++   | ++++  | MIC           | +    | ++   | +++   | ++++  |
| <i>S.treptococcus pyrogenes</i>                 | -                     | -    | -    | -     | -     | -          | -    | -    | -     | -     | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |
| <i>B.acillus subtilis</i>                       | MIC                   | +    | ++   | +++   | ++++  | -          | MIC  | +    | ++    | +++   | MIC      | +    | ++   | +++   | ++++  | MIC           | +    | ++   | +++   | ++++  |
| <i>C.eorynebacterium ulcereum</i>               | -                     | -    | -    | -     | -     | MIC        | +    | ++   | +++   | ++++  | MIC      | +    | ++   | +++   | ++++  | MIC           | +    | ++   | +++   | ++++  |
| <i>E.scherichia coli</i>                        | MIC                   | +    | ++   | +++   | ++++  | -          | MIC  | +    | ++    | +++   | MIC      | +    | ++   | +++   | ++++  | MIC           | +    | ++   | +++   | ++++  |
| <i>Proteus mirabilis</i>                        | -                     | -    | -    | -     | -     | MIC        | -    | -    | -     | -     | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |
| <i>Proteus P. vulgaris</i>                      | -                     | -    | -    | -     | -     | -          | -    | -    | -     | -     | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |
| <i>Pseudomonas aeruginosa</i>                   | -                     | -    | -    | -     | -     | -          | -    | -    | -     | -     | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |
| <i>Salmonella S. typhi</i>                      | MIC                   | +    | ++   | +++   | ++++  | -          | MIC  | +    | ++    | +++   | MIC      | +    | ++   | +++   | ++++  | MIC           | +    | ++   | +++   | ++++  |
| <i>Klebsella K.pneumoniae</i>                   | MIC                   | +    | ++   | +++   | ++++  | -          | MIC  | +    | ++    | +++   | -        | MIC  | +    | ++    | +++   | MIC           | +    | ++   | +++   | ++++  |
| <i>Candida C. albicans</i>                      | -                     | -    | -    | -     | -     | MIC        | -    | -    | -     | -     | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |
| <i>Candida C. stillatoidae</i>                  | -                     | -    | -    | -     | -     | -          | -    | -    | -     | -     | -        | -    | -    | -     | -     | -             | -    | -    | -     | -     |

|                                            |   |   |   |   |   |     |   |    |     |      |     |   |    |     |      |     |   |    |     |      |
|--------------------------------------------|---|---|---|---|---|-----|---|----|-----|------|-----|---|----|-----|------|-----|---|----|-----|------|
| <del>Candida C.</del><br><i>tropicalis</i> | - | - | - | - | - | MIC | + | ++ | +++ | ++++ | MIC | + | ++ | +++ | ++++ | MIC | + | ++ | +++ | ++++ |
|--------------------------------------------|---|---|---|---|---|-----|---|----|-----|------|-----|---|----|-----|------|-----|---|----|-----|------|

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Key: - = no turbidity, (No growth), + = turbidity (Scanty colonies growth), ++ = (Moderate growth), +++ = (High growth), ++++ = (v. heavy colonies growth)

184

MBC / MFC = Minimum Bactericidal Concentration / Minimum Fungicidal Concentration.

185 In Table 2 most of the microbes showed sensitivity to the extracts though at different levels of inhibition  
186 but for *Streptococcus* pyrogenes, *P.roteus* vulgaris, *P.seudomonas* aeruginosa and the fungus *C.andida*  
187 *stellatoidea*, where the extracts do not show any inhibition zones. Petroleum ether extract was seen not to  
188 be active on most of the pathogens e.g. Methicillin Resistant *S.taphylococcus* aureus, or (Methicillin  
189 R.S.A), *S.treptococcus* pyrogenes, *C.orynebacterium* ulcereas, *P.roteus* mirabilis, *P.roteus* vulgaris,  
190 *P.seudomonas* aeruginosa and the fungi, *C.andida* albicans, *C.andida* stellatoidea and *C.andida* tropical.

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191 Thus one can also infer from the table that the chloroform extract has the highest activity, followed by the  
192 ethyl acetate, then the methanol and lastly the petroleum ether. [\(write the full name only in the list of](#)  
193 [strains section\)](#)

194 It was observed from Table 3 that the minimum inhibitory concentration (MIC) was 1.25 mg/mL  
195 ([µg/mL?](#)) generally. This result, also showed that, the chloroform fraction had the highest anti-microbial  
196 reactivity (at this concentration, it has the widest spectrum of affected microbes) followed by the ethyl  
197 acetate fraction, the methanol and lastly the petroleum ether fraction of the stem bark of the plant. This  
198 shows that the secondary metabolites from the plant are highly active on the tested microorganisms. The  
199 present investigation clearly reveals the antibacterial / anti-fungal nature of this plant and portrays it as a  
200 potential source of useful drugs thereby suggesting that it could be exploited in the management of  
201 diseases caused by these bacteria /fungi in human and plant systems (Raghavendra et al., 2006).

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202 MBC /MFC, (Table 4), were carried out to check whether the bacteria and the fungi were killed or only  
203 their growth was inhibited. The average value was 2.25mg/mL ([µg/mL?](#)). MFC was found to be  
204 5.00mg/mL ([µg/mL?](#)) and was effective for chloroform, ethyl-acetate and methanol

205 Currently, no single drug is effective for the treatment of multidrug resistant malaria and combination  
206 therapy includes artemisinin derivatives such as artesunate (David *et al.*, 2004) or mixtures with older  
207 drugs such as atovaquone (Deprez-Poulain and Melnyk, 2005), proguanil (Jones and Good, 2006)  
208 combination malarone / maldox ([Taylor and white, 2004](#); Winter *et al.*, 2006; [Taylor and white, 2004](#)).

209 Unfortunately, first report on drug resistance to arteminin-derivatives (Jambou *et al.* 2005) and to drug  
210 combination therapies (Wichmann *et al.*, 2004) have already appeared. So, in the absence of a functional,  
211 safe and widely available malaria vaccine, efforts to develop new anti-malaria drugs continue.

## 212 CONCLUSION (should be more relevant to the findings)

213 Present study showed that, the stem bark of *N. diderichii* contains an antimicrobial flavanone as one of its  
214 constituents. The antimicrobial activity by *N. diderichii* is significant and may serve as a lead towards the  
215 development of more potent, safe and cost effective antimicrobial agents. It also validates the ethno  
216 medicinal use of the plant in the treatment of various bacterial infections, including typhoid, diarrhoea  
217 and may be enhanced to cure even the current Ebola. To our knowledge, this is the first reported  
218 methylated flavone from *N. diderichii*.

219 The present work should be scaled- up and further work carried out with it on the malaria parasite to  
220 confirm whether the active metabolites would be effective singly or in synergy.

221 Clinical and nutritional studies are recommended for the above indigenous plants so as to be able to  
222 advice locals in the various locations of the ill effects of the plants. Equally, the phytochemistry of these  
223 plants from different locations could be equally compared and research should be carried out on other  
224 parts of the plants to ascertain whether the entire plant possess medicinal properties.

## 225 REFERENCES (references should be fixed as per guidelines of the journal; there variations in 226 formatting)

- 227 Ahmed I, Beg AZ (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against  
228 multi-drug resistant *Stapylococcus aureus*. J. Ethnopharmacol. 74:113-123.
- 229 Cragg GM, Newmann DJ (2005). Plants as a source of anti-cancer agents. J. Ethnopharmacol. 100: 72-79.
- 230 David A. F, Philip J. R, Simon R. C, and Solomon N (2004): Anti-malaria drug discovery:  
231 efficacy models for compounds screening. *Nature Rev.*, 3: 509 -520.  
232
- 233 Deprez-Poulain R, and Melnyk P (2005): 1, 4-Bis (3-aminopropyl)-piperazine libraries: From the  
234 discovery of classical chloroquine –like anti-malaria to the identification of new targets.

235 Comb. Chem.Screen. 8: 39 -48  
 236 Eja M E., Asikong B E., Abriba C., Arikpo G E., Anwan E E and Enyi-Idoh K. H.(2007):  
 237 Comparative Assessment of the Antimicrobial Effects of Garlic (*Allium sativum*) and  
 238 antibiotics on Diarrhoeagenic organisms. *The Southeast Asian journal of tropical Medicine and*  
 239 *Health*: 38 (2): 343 -348.  
 240 Emokpae, M. A; Nwokedi, E. E; and. Dutse, A. I; (2006): Biochemical changes in adult Nigerians with  
 241 pulmonary tuberculosis in Kano-Nigeria *Highland Medical Research Journal* 4(1): 15-21.  
 242 European Centre for Disease Prevention and Control ECDC (2012): Antimicrobial resistance surveillance  
 243 in Europe (2012) Annual report of the European Antimicrobial Resistance Surveillance Network  
 244 (EARS-Net) [www.ecdc.europa.eu](http://www.ecdc.europa.eu)  
 245  
 246 Gupta S S (1994): Prospects and prospective of natural plants products in medicine. *Indian J. pharm*:  
 247 26: 1 -13.  
 248 Jambou R, Legrand E, Niang M, Khim N and Lim p (2005): Resistance of *Plasmodium*  
 249 *falciparum* field isolates to *in vitro* artemether and point mutation of the SERCA type Pf  
 250 ATPase. *Lancet*, 366: 1960 – 1963.  
 251  
 252 Jain, P., and Bari, S. (2010): Stigmasterol and Campesterol from Petroleum ether extra of woody  
 253 stem of *Wrightia tinctoria*. *Asian Journal of Plant Science*, 163-167.  
 254  
 255 Jones M. K and Good m. F (2006): Malaria parasites up close *Nat. Med.* 12: 170 -171  
 256  
 257 Lamidi, M., E. Ollivier, R. Faure, L. Debrauwer, L. Nze-Ekekang, and G. Balansard. (1995)  
 258 : Quinovic acid glycosides from *Nauclea diderichii*. *Planta Med.* 61:280–281.  
 259 Meurer-Grimes B, Macbeth DL, Hallihan B, Delph S (1996). Antimicrobial activity of medicinal plants  
 260 of the scrophulariaceae and acanthaceae. *Int. J. Pharmacog.* 34:243-248.  
 261 Nwankwo, E. K., Kwaru, A; Ofulu, A; and Babashani, M; (2005): Haematological Changes in  
 262 Tuberculosis in Kano, Nigeria *Journal of Medical Laboratory Sciences* 14(2):35-39  
 263  
 264 Raghavendra M .P, Satish S, Raveesha K A (2006): Phytochemical analysis and antibacterial  
 265 activity of *Oxalis corniculata*; a known medicinal plant Agriculture Microbiology Laboratory,  
 266 Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore, 570 006,  
 267 India. (mySCIENCE 1(1), 72–78 °c University of Mysore <http://myscience.uni-mysore.ac.in>

268  
 269 Silver LL, Bostian KA (1993). Discovery and development of new antibiotics: the problem of antibiotic  
 270 resistance. *Antimicrob. Agents Chemother.* 37:377-383.  
 271  
 272 Taylor W. Rand White N. J (2008): Anti-malaria drug toxicity: A review. *Drug Saf.*, 27: 25 -61.  
 273  
 274 Tinto, W., Blair, L., Reynolds, W., and Macleans, S. (1992): The phytochemistry of the flora of  
 275 Qatar Kingprint of Richmond. *Journal of Natural Products*, p.395.  
 276  
 277 Vollekova, A., Kostalova, S., and Sochorova, R. (2001): Isoquinoline Alkaloids from *Mahonia*  
 278 *aquifolium* stem bark is active against *Malassezia Sp.* *Folia Microbiology*, 46, 107-111.  
 279  
 280  
 281 Wichmann O. M, Muhlen H, Grubh, Mockenhaupt F. P, Suttorp N and Jelinek T (2004):  
 282 Malarone treatment failure not associated with previous described mutations in the  
 283 cytochrome gene. *Malaria J.*, 3: 14 -14.  
 284  
 285 Winter R. W, Kelly J. X, Smiikstein M. J, Dodean R, and Bagby G. C (2006): Evaluation  
 286 and lead optimization of anti-malaria acridones. *Exp. Parasitol.*, 114: 47 -56.