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Antimicrobial Activity and Isolation of Methylated Flavanone from Methanol Stem-bark Extract of *Nauclea diderrichii*

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Authors' contributions

This work was carried out in collaboration between all authors. Author MEK designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors MEK and JOA performed the statistical analysis. Authors MEK and IYS managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The antimicrobial activities of the crude methanol fraction of the stem bark extract of *Nauclea diderrichii* was investigated using 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 below detectable limits (BDL) in methicillin resistant *S. aureus, Bacillus subtilis, Corynobacterium ulcerans, Escherichia coli* and the fungus *Candida albicans. L*ow values for minimum inhibitory concentration (MIC, 1.25 µg/ml), and minimum bactericidal concentration (MBC, 2.25 µg/mL), suggested that the crude extract had a good antimicrobial activity against the susceptible organisms. The minimum fungicidal concentration MFC, was 5.00 µg/mL.Chromatographic separation using a combination of silica and sephadex LH-20 led to the isolation of a methylated flavanone. The structure of this isolated compound was

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determined using different spectroscopic techniques including both 1 and 2D nuclear magnetic resonance (NMR). All the above validates the ethno medicinal uses of the *N. diderichii* in the treatment of various bacterial infections.

Keywords: Antimicrobial activity; Nauclea diderrichii; spectroscopic analyses; microbes and secondary metabolites.

1. INTRODUCTION

Bioactive plant secondary metabolites have been found to have a wide variety of micro-organisms and have shown great promise in the treatment of many intractable diseases like the opportunistic infectious diseases. Most of the plant secondary metabolites have been investigated for their chemical components and some of the isolated compounds have been shown to possess diverse and interesting biological activities [1,2,3].

Malaria, diarrhoea and dysentery are health challenges [4,5] and are believed that *Nauclea diderrichii* possesses medicinal properties effective in their management since decoctions of this plant are used for their cure in Mubi North and South LGAs of Adamawa State, North-Eastern Nigeria.

Nauclea diderrichii (De Wild & Dur) Meril (Rubiaceae). Its commercial name is '*bilinga*' and common name is 'African peach', is reputed (known) for its numerous medicinal uses as a tonic and fever medicine (antipyretic!), chewing stick, toothaches; dental caries, septic mouth, malaria, diarrhoea and dysentery cure [6].

Most European countries similarly witness a seemingly unimpeded increase of antimicrobial resistance in the major Gram-negative pathogens which could unavoidably lead to loss of therapeutic treatment options (European Centre for Disease Prevention and Control [7].

Natural products have been the most significant source of drugs and drug leads in history [8]. The emergence of multidrug resistance in human and animal pathogenic bacteria as well as undesirable side-effects of certain antibiotics has triggered immense interest in the search for new antimicrobial / antifungal / antiviral drugs of plant origin [9]. In theory, bacteria will continue to develop resistance once exposed to any antimicrobial agents, thereby imposing the need for a continuous search, develop and advancement of new drugs [10] and higher plants represent a potential source of novel antibiotic prototypes [11].

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

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

2. MATERIALS AND METHODS

2.1 Identification, Collection and Preparation of Plant

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

2.2 Extraction

About 450 g of the powdered stem bark was accurately weighed and percolated with 10 L for 72 hours. After which there was decantation, filtration, and concentration using a rotary evaporator (R110) at 35℃ to obtain the methanol soluble fractions, (F_m01), labelled, $F_{m0}R$, [30.5 g] and this was kept in the refrigerator for further separation. It was later macerated with different solvents of increasing polarities starting with petroleum ether, chloroform, ethyl-acetate and methanol to have their corresponding fractions.

2.3 Thin Layer Chromatography (TLC)

The ethyl-acetate soluble portion of the methanol extract of *N. diderichii,* was subjected to TLC using pre-coated aluminum plates. The solvent systems used were ethyl-acetate: methanol (9:1). The spots on TLC were visualized under ultraviolet (UV) light, spraying with 5% sulphuric acid in methanol followed by heating at a temperature of 110℃ for 5 minutes.

2.4 Chromatographic Separation

2.4.1 Column chromatography, silica gel (60 - 80)

Ethyl acetate soluble fraction (2.5 g) was mounted on a glass column (7.5 × 3.5 cm). The column was eluted continuously using petroleum ether, ethyl acetate and methanol mixture by gradient elution technique; 20 mL each was collected as eluates and the progress of elution was monitored using TLC. A total of 15 fractions (ND-01- ND-15) from 200 collections were made; similar fractions were combined and further purified by Sephadex LH-20 gel filtration column chromatography. In both cases, the progress of separation was monitored using TLC.

2.4.2 Gel filtration

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

2.4.3 Structure determination

Spectral structure determination was conducted at the School of Chemistry, University of Kwazulu-Natal, South Africa using UV light at 356 nm, infra-red (IR) was recorded at 3239.9 cm^{-1}, NMR was run with 400 MG-HtzBruker NMR machine and data confirmed using literature and then MestReNova$^{(R)}$ [12].

2.5 Antimicrobial Assay

2.5.1 Microorganisms/strains

The microorganisms tested included *Staphylococcus aureus,* methicillin resistant *S. aureus, Streptococcus pyogenes, Bacillus subtilis, Corynobacterium ulcerans, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia, Salmonella typhi* and *Candida albicans*. All the organisms were clinical isolates obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria.

2.5.2 Reference drug

Sparfloxacin mcg/disc made by Himedia Laboratories Pvt. Ltd. Mumbai, India was used.

2.5.3 Susceptibility studies

Preliminary antimicrobial activity of the compound was carried out using stock concentration of 20 µg/mL. The microorganisms were maintained on agar slant. The inocula were prepared by inoculating the test organisms in nutrient broth and incubated them for 24 h at 37℃ for the bacteria, while for *C. albicans*, Sabouraud dextrose broth was used and was incubated for 48 h. After incubation, the broth cultures were diluted to 1:1000 for the Gram-positive bacteria and 1:5000 for the Gram-negative bacteria. One milliliter of the diluted cultures was inoculated into a sterile molten nutrient agar at 45℃ and poured into sterile petri-dish. Similarly, 1 mL of the diluted fungal suspension was poured into sterile Sabouraud dextrose agar plates and the excess sucked up with Pasteur pipette. These were swirled gently and allowed to solidify. Wells were bored into the solidified inoculated nutrient agar plates using cork borer of 6 mm diameter. The wells were filled with 0.1 mL of the compound. Sparfloxacin standard disc was also placed on the agar plate. A one hour was allowed for the compound to diffuse into the agar after which the plates were incubated overnight at 37 and 25℃ for bacteria and fungi, respectively. At the end of incubation period, diameter of inhibition zone was measured using transparent ruler and recorded. The compound and standard antibiotic were tested in duplicate and mean zones of inhibition were computed [13].

2.6 Minimum Inhibitory Concentration (MIC)

MIC was determined using broth dilution method [14], half-fold serial dilutions of the compound were made to obtain concentrations of 5.00, 2.50, 1.25, 0.625 and 0.312 µg/mL. 0.2 mL suspension of standard inoculum of each organism was inoculated to the different concentrations of the compound. The test tubes were then incubated at 37℃ for 24 h after which they were observed for the growth. Inhibition of

growth was indicated by a clear solution. The MIC was defined as the lowest concentration of the compound inhibiting the visible growth of each microorganism [15].

2.7 Minimum Bactericidal Concentration (MBC) / Minimum Fungicidal Concentration, (MFC)

The contents of the MIC tubes and the preceding tubes in the serial dilution were sub-cultured into the appropriately labeled nutrient agar plates by dipping a sterile wire loop into each test tube and streaking the surface of the labeled nutrient agar plates. The plates were then incubated at 37℃ for 24 h after which they were observed for colony growth. The lowest concentration of the sub-culture with no growth was considered as MBC / MFC [14].

3. RESULTS AND DISCUSSION

3.1 Extraction

The crude methanol extract (30.5 g) of *N. diderichii* stem bark after extraction, was macerated with petroleum ether, chloroform and ethyl acetate to give the corresponding residues and weights (Table 1).

3.2 Spectral Data

3.2.1 Ultraviolet (UV)

In ND-06, the UV absorption band using a 356 nm UV spectral lamp, giving a white spot is indicative of weak auxochromes that are pH dependent like hydroxyl group (OH). The C=C stretching frequency of the cyclic molecule could be seen at 1604 cm^{-1}.

3.2.2 Infrared (IR)

The IR spectra of the isolated compound indicated prominent absorption frequencies characteristic of certain functional groups: The band of medium intensity at 3239.9 cm^{-1} suggests the presence of OH. The broad band of very strong intensities at 2950 and 2852.76 cm^{-1} are characteristic of C-H stretching of aliphatic groups e.g. CH_3, CH_2, and CH etc. Overtones and combination tones of lower and weak frequency bands are displayed at 1530.8 cm^{-1}. Their appearance and position in a spectrum can be gainfully used for identification of some structural features. The C-H bending vibrations of the CH_3 and CH_2 groups are observed at 1429.6 and 1359.0 cm^{-1}.

3.3 Nuclear Magnetic Resonance (NMR)

3.3.1 Proton (^1H)

The chemical shift of any proton is determined by its chemical environment: The cluster of methyl and methylene protons as observed in the spectra suggests the flavanonal nature of the compounds. DEPT, 90, 45 and 135, provides a clue to the various methyls, methylenes, methines and quaternary carbons. The hydroxyl proton is again confirmed at 4.0969 ppm in the proton NMR spectral.

3.3.2 Carbon (^{13}C)

Compound NDK12: Spectra data of ^1H, ^{13}C, HSQC, HMBC, COSY, DEPT, NOSEY and LC-MS spectroscopy confirm the structure to be a methylated flavanone (Fig. 1), and this was confirmed from literature and further justified by ^{13}C NMR data simulated by MestReNova$^{(R)}$ ^{13}C were within ± 03 ppm [16,17,18].

The NMR spectrum (Fig. 1) indicated thirteen quaternary, three methine, seven methylene, and five methyl carbons and there are other side groups attached on the flavanone skeleton. The hydroxyl moiety is further confirmed by the proton nuclear magnetic resonance ^1H-NMR by the band at 4.7350 ppm. The appearances and positions of other combination peaks /bands in the spectrum have been gainfully used for identification of some structural features.

Table 1. Extractive values of macerated methanol extract of *N. diderichii* stem bark

Extract	Colour	Weight (g)	Recovery yield (%)
Pet. ether	Light brown	2.00	10.52
Chloroform	Pale yellow	4.40	17.80
Ethyl-acetate	Green	3.6	15,65
Methanol	Brown	20.2	28.70

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

Micro-organisms	Zones of inhibition (mm)						
	Pet. ether	**Chloroform**	**Ethyl act.**	**Methanol**	**Sparflo**	**Flocona**	**DMSO**
Methicillin *R.S.A*	-	25 ± 0.48	-	21 ± 0.68	33 ± 0.68	-	-
Staphylococcus aureus	15 ± 0.11	27 ± 0.76	21 ± 0.63	22 ± 0.46	35 ± 0.38	-	-
Streptococcus pyrogenes	-	-	-	-	32 ± 0.67	-	-
Bacillus sublilis	14 ± 0.32	26 ± 0.72	20 ± 0.82	23 ± 0.85	40 ± 0.64	-	-
Corynebacterium ulcereas	-	24 ± 0.43	20 ± 0.84	21 ± 0.73	28 ± 0.74	-	-
Escherichia coli	17 ± 0.41	27 ± 0.62	21 ± 0.50	22 ± 0.62	30 ± 0.46	-	-
Proteus mirabilis	-	22 ± 0.91	-	-	28 ± 0.54	-	-
Proteus volgaris	-	-	-	-	28 ± 0.47	-	-
Pseudomonas aeruginosa	-	-	-	-	28 ± 0.56	-	-
Salmonella typhi	17 ± 0.51	27 ± 0.76	20 ± 0.85	21 ± 0.85	32 ± 0.55	-	-
Klebsiella pneumoniae	19 ± 0.81	25 ± 0.82	21 ± 0.54	25 ± 0.63	40 ± 0.45	-	-
Candida albicans	-	2 ± 0.32	-	-	-	24 ± 0.65	-
Candida stllatoidea	-	-	-	-	-	27 ± 0.78	-
Candida tropicalis	-	24 ± 0.71	20± 0.56	21 ± 0.65	-	21 ± 0.36	-

Results: Mean of three trials ± Standard error, (-) = Resistant Strain, Sparflo = Sparfloxacin (Bacteria control): 10 µg/mL Flucona = Fluconazole (Fungal control): 20 µg/mL

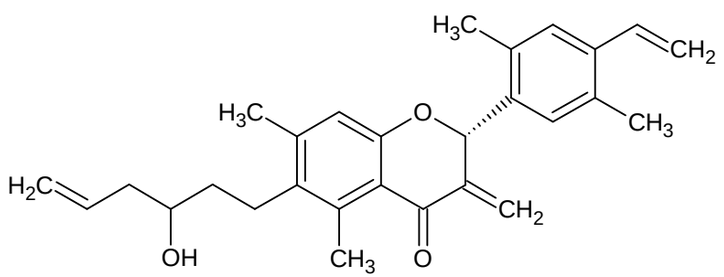

Fig. 1. Suspected structure (A methylated flavanone)

Table 3. Minimum inhibitory concentration (MIC) of petroleum ether, chloroform, ethyl acetate and methanol extracts of the stem bark of *N. diderichii* on fourteen micro-organisms

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	+	++	-	-	-	-	-
S. aureus	5.00	+	+	++	+++	-	-	1.25	++	+++	-	-	1.25	+	++	-	2.50	+	++	++
S. pyrogenes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. sublilis	5.00	+	+	++	+++	-	-	1.25	+	++	-	2.25	-	+	++	-	-	1.25	+	++
C. ulcereas	-	-	-	-	-	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++
E. coli	-	2.25	+	++	+++	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++
P. mirabilis	-	-	-	-	-	-	-	1.25	+	++	-	-	-	-	-	-	-	-	-	-
P. volgaris	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. typhi	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++
K. pneumoniae	-	2.25	+	++	+++	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++
C. albicans	-	-	-	-	-	-	-	1.25	-	-	-	-	-	-	-	-	-	-	-	-
C. stllatoidae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. tropicalis	-	-	-	-	-	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++

Key: - = no turbidity, (No growth), + = turbidity (Light growth), ++ = (Moderate growth), +++ = (High growth)

Table 4. Minimum bactericidal concentration (MBC)/ Minimum fungicidal concentration (MFC) of Petroleum ether, chloroform, ethyl acetate and methanol extracts of the stem bark of *N. diderichii* on some fourteen micro-organisms [20]

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. aureus*	-	-	-	-	-	-	-	MIC	+	++	MIC	+	++	+++	+++	-	-	-	-	-
S. aureus	MIC	+	++	+++	++++	-	MIC	+	++	+++	MIC	+	++	+++	++++	MIC	+	++	+++	++++
S. pyrogenes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. sublilis	MIC	+	++	+++	++++	-	MIC	+	++	+++	MIC	+	++	+++	++++	MIC	+	++	+++	++++
C. ulcereas	-	-	-	-	-	MIC	+	++	+++	++++	MIC	+	++	+++	++++	MIC	+	++	+++	++++
E. coli	MIC	+	++	+++	++++	-	MIC	+	++	+++	MIC	+	++	+++	++++	MIC	+	++	+++	++++
P. mirabilis	-	-	-	-	-	MIC	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. volgaris	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. typhi	MIC	+	++	+++	++++	-	MIC	+	++	+++	MIC	+	++	+++	++++	MIC	+	++	+++	++++
K.pneumoniae	MIC	+	++	+++	++++	-	MIC	+	++	+++	-	MIC	+	++	+++	MIC	+	++	+++	++++
C. albicans	-	-	-	-	-	MIC	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. stllatoidae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. tropicalis	-	-	-	-	-	MIC	+	++	+++	++++	MIC	+	++	+++	++++	MIC	+	++	+++	++++

Key: - = no turbidity, (No growth), + = turbidity (Scanty colonies growth), ++ = (Moderate growth), +++ = (High growth), ++++ = (very heavy colonies growth)
MIC = Minimum Inhibitory Concentration

3.3.3 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) (Fig. 1) was confirmed as being present in the plant.

In Table 2 most of the microbes showed sensitivity to the extracts though at different levels of inhibition but for *S. pyrogenes, P. vulgaris, P. aeruginosa*and the fungus *C. stellatoidea,* where the extracts do not show any inhibition zones. Petroleum ether extract was seen not to be active on most of the pathogens e.g. Methicillin Resistant *S. aureus*, or (Methicillin *R.S.A*), *S. pyrogenes, C. ulcereas, P. mirabilis, P. vulgaris, P. aeruginosa* and the fungi, *C. albicans, C. stellatoidea* and *C.tropical*. Thus one can also infer from the table that the chloroform extract has the highest activity, followed by the ethyl acetate, then the methanol and lastly the petroleum ether.

It was observed from Table 3 that the minimum inhibitory concentration (MIC) was 1.25 µg/mL, generally. This result, also showed that, the chloroform fraction had the highest anti-microbial reactivity (at this concentration, it has the widest spectrum of affected microbes) followed by the ethyl acetate fraction, the methanol and lastly the petroleum ether fraction of the stem bark of the plant. This shows that the secondary metabolites from the plant are highly active on the tested microorganisms. The present investigation clearly reveals the antibacterial / anti-fungal nature of this plant and portrays it as a potential source of useful drugs thereby suggesting that it could be exploited in the management of diseases caused by these bacteria /fungi in human and plant systems [19,12].

MBC /MFC (Table 4), earlier reported by Khan [20] were carried out to check whether the bacteria and the fungi were killed or only their growth was inhibited. The average value was 2.25 µg/mL. MFC was found to be 5.00 µg/mL and was effective for chloroform, ethyl-acetate and methanol.

Currently, no single drug is effective for the treatment of multi-drug resistant malaria and combination therapy includes artemisinin derivatives such as artesunate [21] or mixtures with older drugs such as atovaquone [22], proguam [23] combination malarone / maldox [24,25].

Unfortunately, first report on drug resistance to artemisinin-derivatives [26] and to drug combination therapies [27] have already appeared. So, in the absence of a functional, safe and widely available malaria vaccine, efforts to develop new anti-malaria drugs continue.

4. CONCLUSION

The antimicrobial activity by *N. dederichii* is significant and may serve as a lead towards the development of more potent, safe and cost effective antimicrobial agents. It also validates the ethno medicinal uses of the plant in the treatment of various bacterial infections, including typhoid, diarrhoea and may be enhanced to cure other complicated ailments. To our knowledge, this is the first reported methylated flavone from *N. dederichii*.

5. RECOMMENDATIONS

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

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

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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