American Journal of Chemistry and Applications
2017; 4(2): 21-26
http://www.openscienceonline.com/journal/ajca
ISSN:2381-4527 (Print); ISSN:2381-4535 (Online)

Open Science

Antimicrobial Analysis and Structural Elucidation of Active Compounds of *Nauclea latifolia* Stem Extract (Pin Cushion Tree)

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To cite this article

Ijoma K. I., Ajiwe V. I. E., Alisa C. O. Antimicrobial Analysis and Structural Elucidation of Active Compounds of *Nauclea latifolia* Stem Extract (Pin Cushion Tree). *American Journal of Chemistry and Applications.* Vol. 4, No. 2, 2017, pp. 21-26.

Received: October 9, 2016; **Accepted**: October 20, 2016; **Published**: June 12, 2017

Abstract

Antimicrobial analysis and structural elucidation were carried out on the purified stem extract of *Nauclea latifolia*. The Harbone method was used for the extraction. The extracts were separated using a combination of column chromatography and thin layer chromatography, which gave rise to the isolation of two fractions; these fractions were further purified using recrystallization. The melting point of each pure fraction was determined. The purified extracts were subjected to structural elucidation using various spectroscopic techniques which include; FTIR, UV, H^1 NMR, C^{13} NMR, $DEPT135^0$, COSY, TOCSY, HMBC and HSQC. The spectral analysis suggested the presence of Myristic acid and Palmitoleic acid. The antimicrobial analysis (anti fungal and anti bacterial analysis) using the punched agar diffusion method was carried out on the isolated fractions comparatively with a standard drug Funbact-A cream (a broad spectrum antibiotic). A total of thirteen test organisms were used for this analysis amongst which were ten bacteria test organism and three fungi test organisms. The results from the average diameter zones of inhibition, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentrations (MFC) showed that all the fractions were all active on the entire test organism with zones of inhibition ranging from 10mm-26mm comparatively. None of these fractions showed similar anti microbial effect as the standard drug Funbact-A cream but individually could serve as antimicrobial to diseases caused by these test organisms from their MIC, MBC and MFC.

Keywords

Nauclea latifolia, Antimicrobial Analysis, Structural Elucidation, FTIR, UV-Spectrum, NMR, and Funbact-A Cream

1. Introduction

Compounds which exert various physiological effects of therapeutic value are collectively known as drugs [1]. Herbal medicine sometimes referred to as herbalism or Botanical medicine is the use of herbs for their therapeutic or medicinal value [2]. Out of hundreds of plants species that are recognized as having medicinal values, four out of every five are collected from the wild forest while most of them are from the floras of developing countries [2]. Nearly 50 percent of drugs used in medicine are of plant origin [3]. It has been Shown that *Nauclea latifolia* contained saponine, indole alkaloids and are effective on both bacterial (gram positive and gram negative) and fungal organisms [4]. Also it has been reported that *Nauclea latifolia* can be employed to prevent prolonged menstrual flow [5]. Previous work on *N. latifolia* reported strictosamide, quinovic acid and 3,4-di hydroxy benzoic acid as isolated compounds from the whole root [6], while naucleamides A-E and strictosamide has been isolated from the stem of *N. latifolia* [7].

Therefore, this research is aimed at giving in-depth screening into properties of *N. latifolia* stem that made it useful in the curing of ailments caused by the test organisms

used in this assay since not much is known about its antimicrobial properties and class of active principle.

2. Materials and Methods

2.1. Plant Collection, Identification and Preparation

The stem of *N. latifolia* used in this study was collected from Nnodo Amike-Aba Abakaliki Ebonyi state. It was identified and authenticated as *Nauclea latifolia* by Prof. S.S.C Onyekwelu of the Department of Applied Biology Ebonyi State University. The Fresh stem samples were dried under sunlight; pulverized and stored in a Glass jar for subsequent analysis.

2.2. Extraction and Fractionation into Different Classes

700g of the pulverized stem were soaked in 2800ml and 700ml of methanol:water mixture in a ratio of 4:1 for about 72hour. The mixture was filtered and the filtrate heated on a water bath to one-tenth (1/10) of the initial volume at temperature of about 40°C. The filtrate was then acidified with 2ml of 2M H_2SO_4 and then extracted with chloroform. The mixture was separated using separating funnel. The chloroform extract was heated to dryness and re-dissolved with chloroform giving the chloroform extract [3]. The extract was there after purified using column chromatography and flash chromatography. The process was monitored using preparative thin layer chromatography. The isolated fractions were further recrystalized three times using methanol.

2.3. Anti-bacterial Assay

The sensitivity of the fractions were compared to that of a standard drug (Funbact-A cream) against the selected test organisms (*Bacillus typhi, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella typhi, Staph albus, Staphylococcus aureus, Streptococcus muteus, Aspergillus flavus, Aspergillus niger* and *Candida albican*) was carried out using the punched agar diffusion method [8]. The MIC and MBC were determined using the serial dilution method while MFC was determined using the Punched Agar diffusion method as recommended by Bryant, 1972.

2.4. Structural Elucidation

Molecular formulae and plausible structures were proposed for the two isolated fractions of stem of *N. latifolia* using a combination of spectroscopic techniques such as: FTIR, UV-Visible, 1H, 13C, Dept-135, COSY, TOCSY, HSQC and HMBC NMR analysis

3. Results and Discussions

The results of the organoleptic examination of the leaves of *N. latifolia* stem is shown in Table 1

Table 1. *Results of the Organoleptic Examinations of N. latifolia stem.*

PARAMETER	FRESH STEM	DRIED STEM
COLOUR	Green	Brown
ODOUR	Odourless	Pungent
TASTE	slightly bitter	slightly better

The presence of a bitter taste in a plant tissue indicates the ability of the plant to tone vital organs especially liver and kidney [9].

Table 2. *TLC Results of N. latifolia Stem Crude extracts.*

PARAMETER	R_f VALUE	SOLVENT SYSTEM
N. latifolia stem fraction 1	0.660	Chloroform: methanol (80:5)
N. latifolia stem fraction 2	0.233	Chloroform: methanol (80:5)

The TLC results showed two spot which was later resolved using a solvent system of petroleum ether and chloroform which gave two distinct colours under iodine vapour with R_f values as shown in Table 3

Table 3. *TLC results of N. latifolia stem pure extracts.*

PARAMETER	R_F VALUE	SOLVEN T SYSTEM
N. latifolia stem fraction 1	1.452	Petroleum Ether: Chloroform (4:1)
N. latifolia stem fraction 2	0.513	Petroleum Ether: Chloroform (4:1)

The resulting fractions were recrystallized three times using methanol; this afforded a white crystalline solid and a colourless liquid.

Table 4. *Melting point Results of N. latifolia stem pure extracts.*

PARAMETER	MELTING RANGE (°C)
N. latifolia stem fraction 1	54°C-56°C
N. latifolia stem fraction 2	-

The melting points were uncorrected for all the extracts. The melting temperature range of any chemical analysis is an indication of the extent of purity, hence can be used to estimate whether a substance is pure or contaminated. The melting point of a pure unknown compound is sharp and ranges from 0°C-5°C [10].

Table 5. *Results of MIC and MBC for Funbact-A, N. latifolia stem fraction 1, and N. latifolia stem fraction 2.*

extract Solvent	*E. coli* NCTC 10418	*S.aureus* NCTC 6571	*Bacillus Specie* L.C.I	*Proteus Vulgaris* L.C.I	*Salmonella typhi* L.C.I	*Pseudomonas aeroginosa* L.C.I	*Klebsiela Pneumonia* L.C.I	*Staph. Albus* L.C.I	*Enterobacter Aerogenes* L.C.I	*Strept Mutans* L.C.I
Funbact-A MIC	1:32	1:64	1:16	1:64	1:32	1:32	1:32	1:64	1:64	1:32

extract Solvent	*E. coli* NCTC 10418	*S.aureus* NCTC 6571	*Bacillus Specie* L.C.I	*Proteus Vulgaris* L.C.I	*Salmonella typhi* L.C.I	*Pseudomonas aeroginosa* L.C.I	*Klebsiela Pneumonia* L.C.I	*Staph. Albus L.C.I*	*Enterobacter Aerogenes* L.C.I	*Strept Mutans* L.C.I
MBC	1:16	1:32	1:8	1:32	1:16	1:16	1:16	1:32	1:32	1:16
N. latifolia Stem fraction 1										
MIC	1:4	1:8	1:4	1:16	1:4	1:4	1:4	1:16	1:32	1:4
MBC	1:2	1:4	1:2	1:8	1:2	1:2	1:2	1:8	1:16	1:2
N. latifolia Stem fraction 2										
MIC	1:4	1:8	1:4	1:16	1:4	1:4	1:4	1:16	1:32	1:4
MBC	1:2	1:4	1:2	1:8	1:2	1:2	1:2	1:8	1:16	1:2

The results of the MIC and MBC for *N. latifolia* stem fraction 1, *N. latifolia* stem fraction 2 and Funbact-A cream was shown in Table 5. The MIC values was minimum at 3.125mg/ml and maximum at 25mg/ml for both *N. latifolia* stem fraction 1 and *N.latifolia* stem fraction 2 while the MBC value was minimum at 6.25mg/ml and 50mg/ml for both *N. latifolia* stem fraction 1 and *N. latifolia* stem fraction 2. The minimum inhibitory concentration refers to that concentration which will inhibit 99% or more of the microbe and represents the minimum quantity which will reach the site of infection in order to be useful. The lower the MIC values of a drug the more active the drug is. The MIC and MBC results shows that *N. latifolia* stem fraction 1 and *N. latifolia* stem fraction 2 can serve as a broad spectrum antimicrobial even at a very lower concentration. This confirmed the use of *N. latifolia* stem by ethno medicine practitioners for the treatment of ailments caused by the pathogenic organisms used in this assay.

Table 6. *Results of Average Diameter zone of inhibition for N. latifolia stem fraction 1, N. latifolia stem fraction 2 and Funbact-A Cream for bacteria organisms.*

Extract Solvent	Vol. Used in cm^3	Average diameter (mm) of zones of inhibition on test organism									
		E.coli 10418	*S.aureus* NCTC 6571	*Bacillus Species* L.C.I	*Proteus Vulgaris* L.C.I	*Salmonella Typhi* L.C.I	*Pseudomonas Aeroginosa* L.C.I	*Klebsiella pneumonia* L.C.I	*Staph Albus* L.I.C	*Strept Muteus* L.C.I	*Enterobacter Aerogenes* L.C.I
N. latifolia stem fraction 1	0.05	14	16	12	24	12	14	14	20	12	26
N. latifolia stem fraction 2	0.05	12	16	12	20	10	12	12	18	12	22
Funbact-Acream	0.05	24	32	18	36	22	28	24	34	28	38

Table 6 shows the results of the average diameter zones of inhibition for the bacteria organisms. The Results of the average diameter Zones of inhibition ranges from 12mm-26mm for *N. latifolia* stem fraction 1 and 10mm-22mm for *N. latifolia* stem fraction 2. This indicates that the bacteria organisms were all susceptible to *N. latifolia* stem fraction 1 and *N. latifolia* stem fraction 2; Apart from *Salmonella Typhi* which was resistant to *N. latifolia* stem fraction 2 at 10mm, *N. latifolia* stem fraction 1 and *N. latifolia* stem fraction 2 had intermediate to susceptible antimicrobial properties. The antimicrobial properties of *Nauclea latifolia* stem fractions further confirm the use of *N. latifolia* stem to treat diseases caused by the organisms used in this assay.

Table 7. *Results of Average Diameter Zones of inhibition for N. latifolia stem fraction 1, N. latifolia stem fraction 2 and Funbact-A Cream for fungi organisms.*

Extract Solvent	Volume used in cm^3	AVERAGE DIAMETER (mm) OF ZONES OF INHIBITION ON TEST ORGANISM		
		Aspergillus flavus. L.C.I	*Aspergillus niger*. L.C.I	*Candida albicans* L.C.I
N. latifolia stem fraction 1	0.05	12	12	14
N. latifolia stem fraction 2	0.05	10	10	12
Funbact-A cream+5ml distilled water	0.05	36	36	38

Table 7 shows the results of the average diameter zones of inhibition for the fungi organisms. The results show that *N. latifolia* stem fraction 1 had intermediate to susceptible antimicrobial properties. *Aspergillus flavus* and *Aspergillus niger* were both resistant to *N. latifolia* stem fraction 2 at 10mm for both *Aspergillus flavus* and *Aspergillus niger* while *N. latifolia* stem fraction 2 had an intermediate antimicrobial effect towards *Candida albican* at 12mm. An inhibitory zone diameter of 10mm or less indicates that the organism was resistant, an inhibitory zone diameter of 11-15mm shows intermediate effect while 16mm and above indicates that the organism was susceptible to the compound [11]. Hence *N. latifolia* stem fraction 1 and *N. latifolia* stem fraction 2 had an intermediate effect on the fungi organisms.

Table8. *Results of MIC and MFC for N. latifolia stem fraction 1, N. latifolia stem fraction 2, Funbact-A cream.*

Extract Solvent	*Aspergillus flavus* L.C.I	*Aspergillus niger* L.C.I	*Candida albicans* L.C.I
N. latifolia stem fraction 1			
MIC	1:4	1:4	1:4
MFC	1:2	1:2	1:2
N. latifolia stem fraction 2			
MIC	1:4	1:4	1:8
MFC	1:2	1:2	1:4
Funbact-A CREAM			
MIC	1:64	1:64	1:64
MFC	1:32	1:32	1:32

Table 8 shows the MIC and MFC for the fungi organisms. The MIC and MFC value for *N. latifolia* stem fraction 1 was 25mg/ml and 50mg/ml respectively for all test fungi organisms. *N. latifolia* stem fraction 2 had the MIC and MFC minimum at 12.5mg/ml and 25mg/ml respectively. Also *N. latifolia* stem fraction 2 had the MIC and MFC maximum at 25mg/ml and 50mg/ml respectively. This indicates that *N. latifolia* can serve as antimicrobial even in such a low concentration.

From the results obtained from the antimicrobial analysis, it was confirmed that *N. latifolia* had a good antimicrobial effect on the test organisms comparable to the standard drug Funbact-A cream, and as such its therapeutic benefits can as well be harnessed for the treatment of ailment caused by the organisms used in this assay.

Note: NCTC = National collection of type cultures

L.C.I = Local clinical isolate

Structural elucidations of N. latifolia stem Extracts

Table 9. *FTIR Spectrum Results of N. latifolia Stem Fraction 1.*

wave band (cm^{-1})	Description
675.79, 826.88	CH deformation bonds of alkyl and methyl groups
1407.15, 1513.28	C=O and C-O stretch of acids and esters
2222.38, 2460.49	C-H stretch of alkyl groups
3267.26, 3587.26, 3717.89, 3866.21	OH stretch of alcohols, esters and acids

Table 10. *Results of UV-Visible of N. latifolia Stem Fraction 1.*

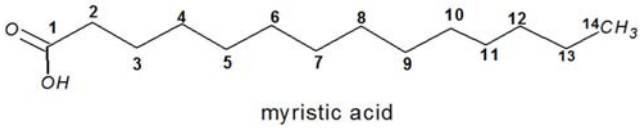
λ max (nm)	CHROMOPHORE DESCRIPTION
760.50	C=O of a acids ($n \rightarrow \pi*$)
449.00, 323.50, 319.00, 305.50, 300.00	C=O of acids ($\pi \rightarrow \pi*$)
270.00, 250.50, 242.50, 235.50, 229.00, 214.00	C=O ($n \rightarrow \pi*$)

Table 11. *Summary of the $H^{1}$and C^{13}Nmr of N. latifolia Stem Fraction 1.*

	EXPERIMENTAL		LITERATURE (Bhunia *et al*, 2015, Wimalasena and Karunawansha, 1994, Hawas, 2014)	
Position	^{1}H	^{13}C	^{1}H	^{13}C
1	-	180.23	-	180.43
2	2.35 (t, 2H)	34.08	2.42 (t, 2H)	34.17
3	1.63 (m, 2H)	24.69	1.51 (m, 2H)	24.74
4	1.26 (m, 20H)	29.39	1.28 (m, 20H)	29.72
5	1.30 (m, 20H)	29.46	1.28 (m, 20H)	29.44
6	1.27 (m, 20H)	29.68	1.28 (m, 20H)	29.67
7	1.33 (m, 20H)	29.04	1.28 (m, 20H)	29.03
8	1.32 (m, 20H)	29.23	1.28 (m, 20H)	29.32
9	1.32 (m, 20H)	29.59	1.28 (m, 20H)	29.51
10	1.31 (m, 20H)	29.27	1.28 (m, 20H)	29.20
11	1.31 (m, 20H)	29.08	1.28 (m, 20H)	29.14
12	1.30 (m, 20H)	31.95	1.28 (m, 20H)	32.00
13	1.30 (m, 20H)	22.72	1.28 (m, 20H)	22.77
14	0.88 (t, 3H)	14.17	0.85 (t, 3H)	14.20

Note: All NMR analysis was carried out in Deuterated chloroform ($CDCl_3$)

The combination of the FTIR, UV-Visible, 1H-NMR, 13C-NMR, DEPT-135°-NMR, COSY, TOCSY, HMBC, and HSQC spectral was matched with previous literature on Myristic acid [12-14] and the structure was proposed as shown below in figure1a



myristic acid

Figure 1a. *Structure of Myristic acid.*

It was previously reported that Myristic acid is an antibacterial and antifungal agent [15-17]. Narasimhan *et al*., (2006) tested the antimicrobial activity of Myristic acid derivatives and presented that Myristic acid derivatives are more effective against gram positive bacteria rather than gram negative bacteria. It is also concluded that S. *aureus* is the most sensitive organism to Myristic acid derivatives [18]. It is also reported that Myristic acid has antifungal activity against *Aspergillus niger* [19, 20], *Candida albicans* [21].

Table 12. *Result of FTIR of N. latifolia Stem Fraction 2.*

wave band (cm^{-1})	Description
711.49, 840.51	C-H deformation bonds for methyl group
916.38	C-H deformation bonds for alkyl groups
1037.77	C-O deformation bonds for acids, alcohols and esters
1262.92	C-O stretch of alcohols, acids and esters
1434.58	C=C stretch for alkenes
1668.10, 1829.53	C=O stretch of aldehyde, acids and esters
2257.43, 2457.72, 2607.80	C=C stretch
2760.28, 2917.23, 3075.27	C-H stretch for alkanes and aromatics
3188.48, 3357.38, 3665.78, 3819.44	OH stretch of alcohols, acids and esters

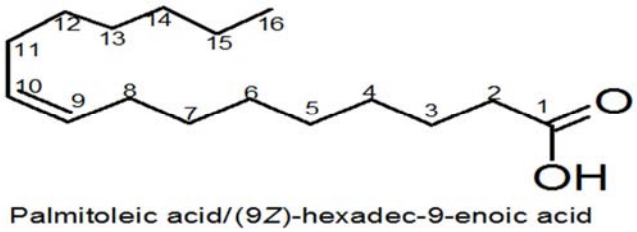
Table 13. *Results of UV-Visible of N. latifolia Stem Fraction 2.*

Λ max (nm)	CHROMOPHORE DESCRIPTION
448.50	C=O of acids ($n \rightarrow \pi*$)
397.50, 319.00, 311.00, 304.50	C=C of alkenes ($\pi \rightarrow \pi*$)
288.00, 274.00, 270.00, 251.00	C=O of acids ($n \rightarrow \pi*$)
242.50, 236.00, 226.50, 221.50, 216.00, 207.00	C=C of alkenes($n \rightarrow \pi*$)

Table 14. *^{1}H and C^{13} NMR Results of N. latifolia Stem Fraction 2.*

	EXPERIMENTAL		LITERATURE (Li *et al.*, 2014)	
POSITION	**^{1}H**	**^{13}C**	**1H**	**^{13}C**
1	-	180.22	-	179.81
2	2.35 (t, 2H)	34.06	2.35 (t, 2H)	34.00
3	1.62 (m, 2H)	24.66	1.63 (m, 2H)	24.67
4	1.27 (m, 16H)	31.80	1.27 (t, 16H)	31.79
5	1.28 (m, 16H)	29.15	1.27 (t, 16H)	29.15
6	1.29 (m, 16H)	29.02	1.26 (t, 16H)	29.04
7	1.29 (m, 16H)	29.00	1.27 (t, 16H)	29.00
8	2.02 (m, 4H)	27.16	2.01 (t, 4H)	27.26
9	5.35 (m, 2H)	129.75	5.33 (t, 2H)	129.76
10	5.36 (m, 2H)	130.06	5.36 (t, 2H)	130.03
11	2.02 (m, 4H)	27.16	2.03 (t, 4H)	27.17
12	1.32 (m, 16H)	29.75	1.27 (t, 16H)	29.75
13	1.31 (m, 16H)	29.68	1.27 (t, 16H)	29.69
14	1.31 (m, 16H)	29.07	1.33 (t, 16H)	29.07
15	1.31 (m, 16H)	22.68	1.34 (t, 16H)	22.67
16	0.89 (t, 3H)	14.14	0.88 (t, 3H)	14.10

The combination of the FTIR, UV-Visible, 1H-NMR, 13C-NMR, DEPT-135^{o}, COSY, TOCSY, HMBC, and HSQC spectral was matched with previous literature on Palmitoleic acid [22]. The structure was proposed as Palmitoleic acid.



Palmitoleic acid/(9Z)-hexadec-9-enoic acid

Figure 1b. *Structure of Palmitoleic acid.*

(*9Z*)-Hexadec-9-enoic acid also known as Palmitoleic acid is an omega-7 mono unsaturated fatty acid that is biosynthesized from palmitic acid by the action of the enzyme delta-9-desaturase. (*9Z*)-Hexadec-9-enoic acid has been shown to increase insulin sensitivity by suppressing inflammation, as well as inhibit the destruction of insulin-secreting pancreatic beta cells [23], the presence of Palmitoleic acid in the stem of *N. latifolia* accounts for its use in the treatment of diabetes by ethno medicine practitioners in Nnodo Amike-Aba, Abakaliki, Ebonyi state.

4. Conclusion

Myristic acid and Palmitoleic acid have been extracted from methanol-water mixture of *N. latifolia* stem. UV, FTIR, *1*D and *2*D NMR spectroscopy as well as comparison with existing literature was used for structural elucidation of the compounds. Antimicrobial (antibacterial and antifungal) analyses showed that the extracts had broad spectrum antimicrobial properties and was comparable to Funbact-A cream a broad antimicrobial agent used in the assay. The ethno medicinal uses of the plant to treat ailments such as ulcers, burns, urinary tract infections, wound infections, sore throat, headache, diarrhea and dysentery caused by human pathogenic organisms have been confirmed.

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