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Research Article

Pharmaceuticals from the whole root of *Nauclea latifolia* (Rubiaceae)

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Abstract

The fruit, leaf, stem and root of *Nauclea latifolia* have been found to posses antimicrobial properties. This work shows the characterization and antimicrobial studies of pharmaceuticals from the whole root of the plant. Soxhlet extraction of the whole root was done using HPLC grade n-hexane, ethylacetate and methanol in that order. Silica gel and sephadex column chromatography were used to isolate pure fractions. Three compounds were isolated. NMR spectroscopic analyses were used to elucidate the structures of the compounds as: strictosamide, quinovic acid and 3,4 – dihydroxybenzoic acid. The compounds were found to have antibacterial and antifungal activities against eleven (11) human pathogenic bacteria namely: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Streptococcus mutans, Streptococcuss pyogenes, Klebsiella pneumonia, Salmonella typhii, Proteus vulgaris, Enterobacter aerogenes, Staphylococcus albus* and *Bacillus subtilis* and three human pathogenic fungi namely: *Candida albicans, Aspergillus flavus* and *Aspergillus niger*. The average diameters of zones of inhibition of the isolated compounds were comparable with those of the standard antibiotics (gentamicin for bacteria and tioconazole for fungi). The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the three compounds confirmed they were active even at low concentrations.

Keywords: *Nauclea latifolia*, pharmaceuticals, chromatographic separations, NMR/Antimicrobial analyses

1. Introduction

Nauclea latifolia is a straggling shrub in thicket in Savanna woodland belonging to the Rubiaceae family. It is found in the forest and fringe tropical forest. It originated from tropical Africa and Asia [1]. It is usually about 7m high but growing up to 35m in closed forests. The wood of *N. latifolia* ('Opepe' wood – Yoruba, Nigeria) is termite resistant and is used as live stakes in farms [2]. The plant has been used as a tonic, fever medicine, in the treatment of severe digestive problem, neurological disarders and infectious diseases, as chewing stick, for toothaches, dental caries, septic mouth, malaria, diarrhea, dysentery and 'jedi-jedi' (pile worm) in children ethnomedically [2-5]

The root has antibacterial activity. It is effective against Gram positive and Gram negative bacteria and antifungal activity against *Corynebacterium diphtheria*, *Streptobacillus* spp., *Pseudomonas aeruginosa*, *Salmonella* spp. [4,6].

Nauclea latifolia contains diverse phytochemicals such as alkaloids, flavonoids, steroids and glycosides. Earlier workers on the plant isolated a series of alkaloids from it. [7-9]

2. Material and Methods

2.1 Plant Collection, Identification and Preparation

The plant was collected in front of the Akanu Ibiam Federal Polytechnic Guest House, Unwana, Afikpo, Ebonyi State. It was identified by Mr. Abel Ngwuta, the Polytechnic horticulturist and confirmed from Okujagu, 2008 as *Nauclea latifolia*. The whole root was sliced, air dried, ground, stored in large sample bottles and labeled.

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2.2 Extraction, Silica gel and Sephadex Column Chromatography of *N. latifolia* whole root

Pulverized *N. latifolia* whole root (600.00g) were exhaustively extracted by the soxhlet method using the solvents: n-hexane (n-Hex), ethylacetate (EtOAc) and methanol (MeOH) of HPLC grade in that order at 40^0C. The extracts were rotary evaporated at 40^0C to dryness. The crude extracts were coded NLRHC (n-Hex crude), NLREC (EtOAc crude) and NLRMC (MeOH crude).

Rotary evaporation of NLREC and the treatment of the filtrate

During the rotary evaporation of NLREC, a yellow solid was deposited. This was transferred to a weighed evaporating dish and was allowed to dry to constant weight in the fume hood and was labelled NLRE ppt 1 (ppt 1from *N. latifolia* whole root EtOAc extract). The supernatant was placed in the freezer and NLRE ppt 2 (ppt 2 from *N. latifolia* whole root EtOAc extract) was obtained. The filtrate gave NLRE ppt 3 (ppt 3 from *N. latifolia* whole root EtOAc extract) when placed in the freezer. The solids were washed with n-Hex, dried, dissolved in MeOH and precipitated with EtOAc. Each gave a bitter taste at the back of the throat when being transferred into the vials. The filtrate was dried, weighed and used for column chromatography. Proton (^{1}H), Carbon – 13 (^{13}C) and two dimensional (2D) NMR analyses were carried out on the three solids in DMSO [10, 11]. JOEL FT NMR spectrophotometer 400 MHz was used for the NMR analyses.

Silica gel Column Chromatography of NLREC filtrate

Dried NLREC filtrate (2.50g) was dissolved in a little MeOH and a little column chromatography (CC) grade silica gel (silica gel 60) was added. This was allowed to dry in the fumehood. The pre-adsorbed extract was transferred to the top of the prepared silica gel 60 column (slurry prepared using n-Hex: EtOAc, 90:10). n-Hex: EtOAc 90:10 was used as the starting solvent mixture. The polarity was increased by 10% up to EtOAc: MeOH, 70: 30 [12]. A total of 105 fractions of 20 ml each were collected. They were allowed to dry in the fumehood and inspected for solid formation. The code was NLRE. NMR analyses were carried out in DMSO.

Sephadex LH – 20 Column Chromatography of NLRMC (methanol crude)

N. latifolia whole root crude methanol extract (2.0g) were dissolved in a little MeOH. The sample solution was loaded into a long-necked Pasteur pipette. The tip of the pipette was held against the wall of the column (prepared using sephadex LH – 20 in MeOH) and moved in a circular motion so that the sample layered into the gel bed. The column was run slowly and the sample components were eluted isocratically using MeOH [13,14].

The code was NLRS. A total of 40 fractions of 5.0 ml each were collected and inspected for solid formation. TLC was done using EtOAc: MeOH (50:50) + 2 drops of ethanoic acid. 5% $FeCl_3$ was used as the spraying reagent. Similar fractions were pooled together. NLRS 19 and 20 were pooled together and labelled NLRS 20. The NMR analysis was done in DMSO.

2.3 Structural Elucidation

^{1}H, ^{13}C and 2D NMR analyses were used for the structural elucidation of the isolated compounds.

2.4 Anti – Microbial Assay

The sensitivity of the three isolated compounds against the eleven human pathogenic bacteria *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Streptococcus mutans, Salmonella typhii, Streptococcus pyogenes, Klebsiella pneumoniae, Proteus vulgaris, Enterobacter aerogenes, Staphylococcus albus* and *Bacillus* subtilis and three human pathogenic fungi namely: *Candida albicans, Aspergillus flavus* and *Aspergillus niger* was carried out using the punched agar diffusion method [15-17]. MIC, MBC and MFC were determined using the serial diffusion method by the same authors. The antimicrobial activities were studied in different concentrations (200, 100, 50, 25, 12.5, 6.25 3.125 mg/ml) using 0.05 ml volume of each.

3. Results and discussion

3.1 Spectroscopic Analysis and Structural Elucidation of the isolated compounds

3.1.1 Identification of Strictosamide

NLRE ppt 1 (1.90g), NLRE ppt 2 (1.85g), NLRE ppt 3(1.50g) were obtained from the ethyl acetate crude extract of *Nauclea latifolia* whole root as yellow solids. They gave a positive Dragendorff's test (reddish-yellow) showing they are alkaloids. They were UV sensitive (254 nm and 364 nm) and gave a yellow-white fluorescence. The solids were separately dissolved in MeOH and precipitated with EtOAc. They had the same m.p., 179^{o}C. They showed similar proton and C-13 spectra indicating they were the same compound. NLRE ppt 2 is thus used in the discussion.

The ^{1}H and ^{13}C NMR analyses were carried out in DMSO-d_6 and the results are shown in Table 1.

Table 1: ^{I}H and ^{13}C NMR spectral data of NLRE ppt 2 in DMSO-d_6, δ (ppm)

Position	^{I}H	^{13}C
1	11.0(1 H, s)	-
2	-	135.02
3	2.98(1H, t)-	42.5
4	-	-
5	4.86(5a,1 H, d)	42.90
	4.90(5b,1 H, dd)	
6	2.58(6a,1 H, dd)	21.12
	2.89(6b,1 H, m)	
7	-	108.40
8	-	126.90
9	7.38(1 H, d)	117.50
10	6.98(1 H, t)	118.60
11	7.07(1 H, t)	121.00
12	7.35(1 H, d)	111.30
13	-	134.00
14	2.05(14a,1 H, m)	25.60
	2.45(14b,1 H, m)	
15	2.60(1 H, m)	23.40
16	-	107.40
17	-	163.70
18	5.35(18a,1 H, d)	119.90
	5.36(18b,1 H, d)	
19	5.58(1 H, m)	133.30
20	2.59(1H, m)	42.80
21	5.33(1H, d)	95.70
22	7.25(1H, d)	146.80
1^1	4.43(1H, d)	99.60
2^1	2.94(1H, s)	70.60
3^1	2.80(1 H, s)	73.40
4^1	3.08(1H, s)	77.20
5^1	3.08(1H, s)	77.70
6^1	3.39(6^1a, 1H, s)	61.89
	3.69(6^1b, 1H, s)	

The ^{1}H NMR spectral data revealed a number of characteristics of the indole alkaloid. The presence of an un-substituted indole ring system was deduced from the signals at δ 7.38, δ6.98, δ7.07 and δ7.35ppm. A vinylidine substituent was indicated by the signals at δ5.35, δ5.36 and δ5.58 ppm. The presence of an O-β-B-glycoside substituent was suggested by the anomeric proton signal at δ4.43, δ3.39 and δ3.69ppm.

The ^{13}C NMR spectral data showed the resonances of all the 26 carbons present in the compound which showed the presence of $5CH_2$, 15CH and 6 quarternary carbons in this compound. The COSY experiment, Figure 2 correlated the coupled protons whereas the HSQC experiment, Figure 3 confirmed the number of protons that were coupled to the carbon atoms.

These spectroscopic data were compared with literature [18-20] and were discovered to be the same for strictosamide (indole alkaloid) and was thus characterized (Figure 1a).

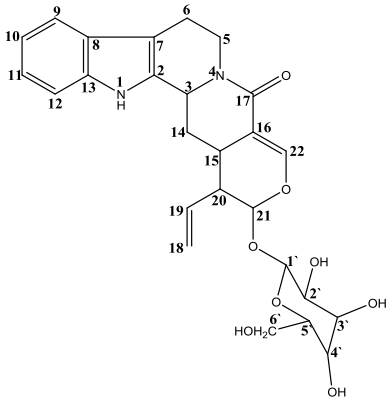

Figure 1a: Structure of Strictosamide

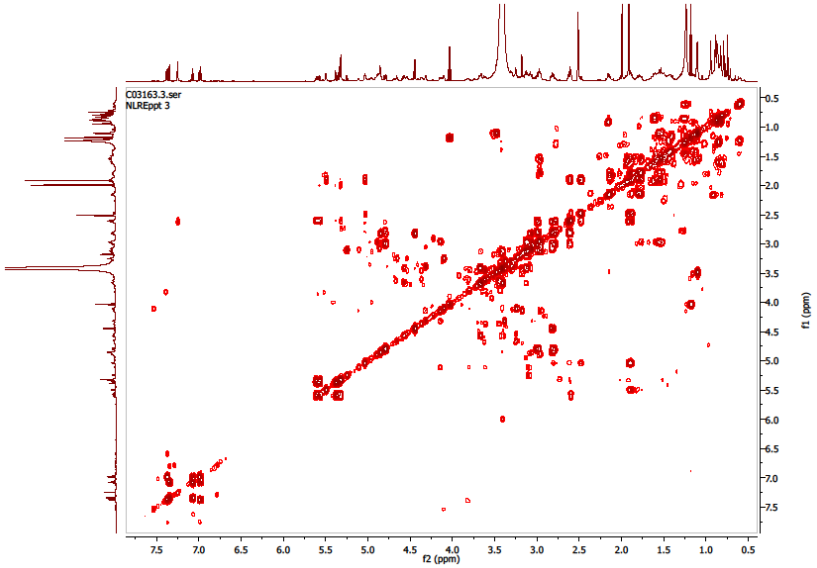

Figure 2: COSY Spectrum of NLRE ppt 2

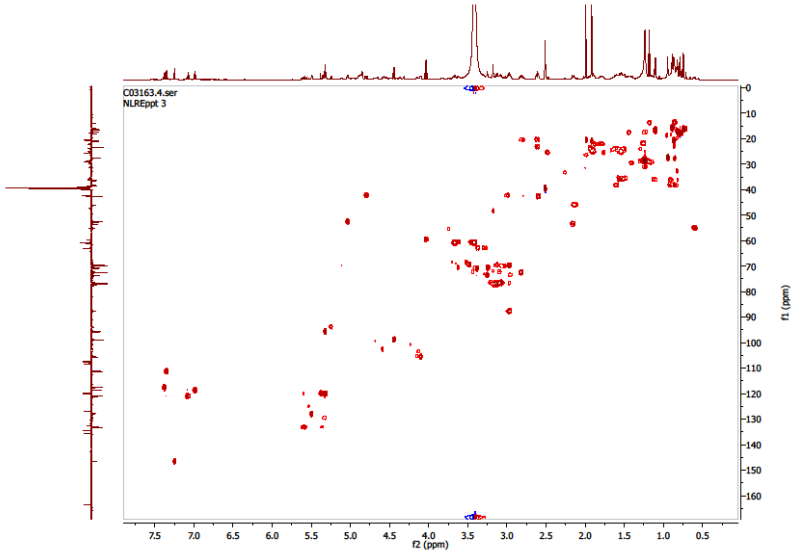

Figure 3: HSQC Spectrum of NLRE ppt 2

3.1.2 Identification of Quinovic acid

NLRE 90 (0.46g) m.p. 296^{0}C (decomposed) was isolated as fraction 90 from column chromatography of ethyl acetate extract of *Nauclea latifolia* whole root (EtOAc: MeOH 70:30) as a pale yellow amorphous powder. It was dissolved in MeOH and precipitated using n-Hex. It was active in UV (254 nm and 364 nm) and gave a purple/pink colour upon treatment with anisaldehyde - H_2SO_4 reagent on TLC on heating.

Proton and Carbon-13 NMR analyses of NLRE 90 were carried out in DMSO-d_6 and the results are shown in Tables 2 and 3.

Table 2: Characteristic ^{1}H NMR spectral data of NLRE 90 in DMSO-d_6, δ (ppm)

	^{1}H
H-3	2.96(1H,t)
H-12	5.50(1H,t)
H-18	2.15(1H,d)
H-23	0.74(3H,s)
H-24	0.95(3H,s)
H-25	0.89(3H,s)
H-26	0.76(3H,s)
H-29	1.10(3H,s)
H-30	1.10(3H,s)

Table 3: Carbon-13 NMR spectral data of NLRE 90 (Quinovic acid) in DMSO-d_6, δ(ppm)

Position	**^{13}C**
1	38.53
2	24.97
3	88.30
4	39.10
5.	55.60
6	18.93
7	36.90
8	39.30
9	46.50
10	36.70
11	22.60
12	128.50
13	132.90
14	55.80
15	30.40
16	31.34
17	47.95
18	54.10
19	36.85
20	38.70
21	30.00
22	30.26
23	23.06
24	21.08
25	16.20
26	17.30
27	178.40
28	176.7
29	17.90
30	21.00

In the ^{1}H NMR spectral data indicated in Table 3.0, 6 methyl protons which resonated at 0.74, 0.95, 0.89, 0.76, 1.10 and 1.10 ppm, the highly deshielded proton at 5.50 ppm suggested a compound in the urs-12-ene series.

The appearance of highly deshielded carbons in Table 2.0 at 128.50, 132. 90,178.40 and 176.7 ppm also indicated an urs-12-ene dioic acid. COSY experimental result, Figure 4 correlated the coupled protons while the HMBC, Figure 5 and HSQC, Figure 6 corroborated the number of coupled protons to the carbon skeleton. These observations and in comparison with literature [21-26] established NLRE 90 as quinovic acid (triterpene hydroxy-dicarboxylic acid of the urs -12-ene type) and is thus confirmed (Figure 1b).

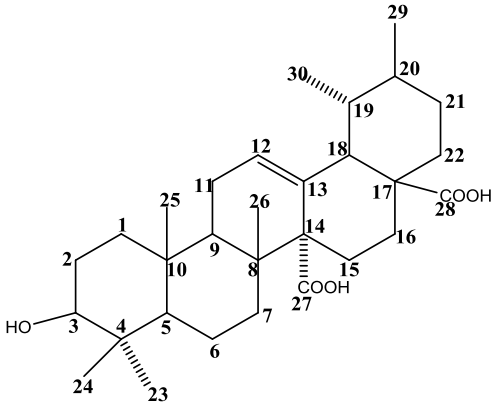

Figure 1(b): Structure of Quinovic acid

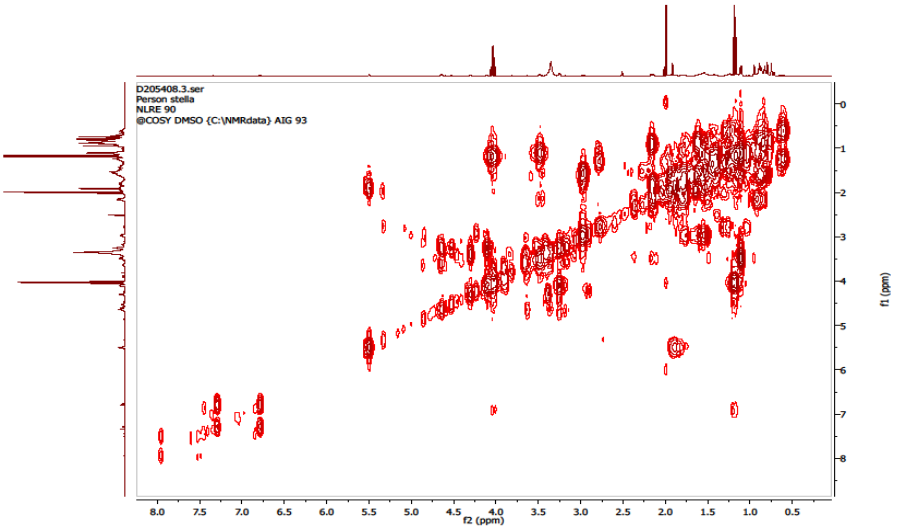

Figure 4: COSY Spectrum of NLRE 90

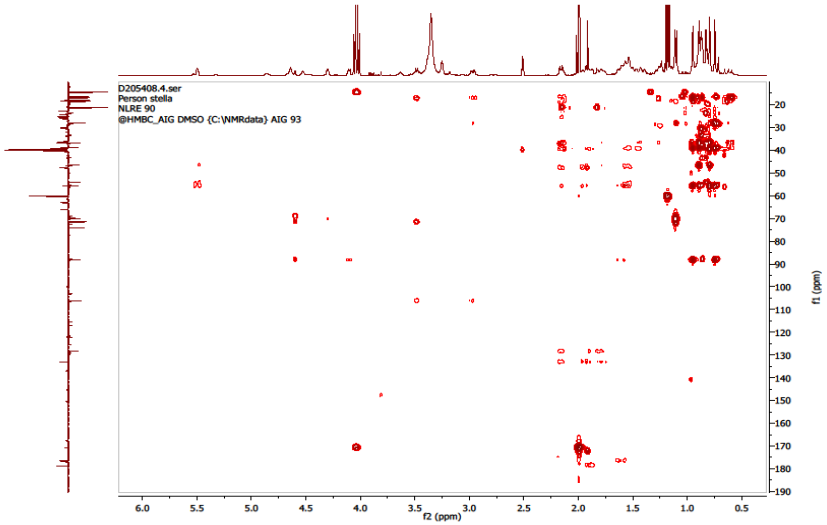

Figure 5: HMBC Spectrum of NLRE 90

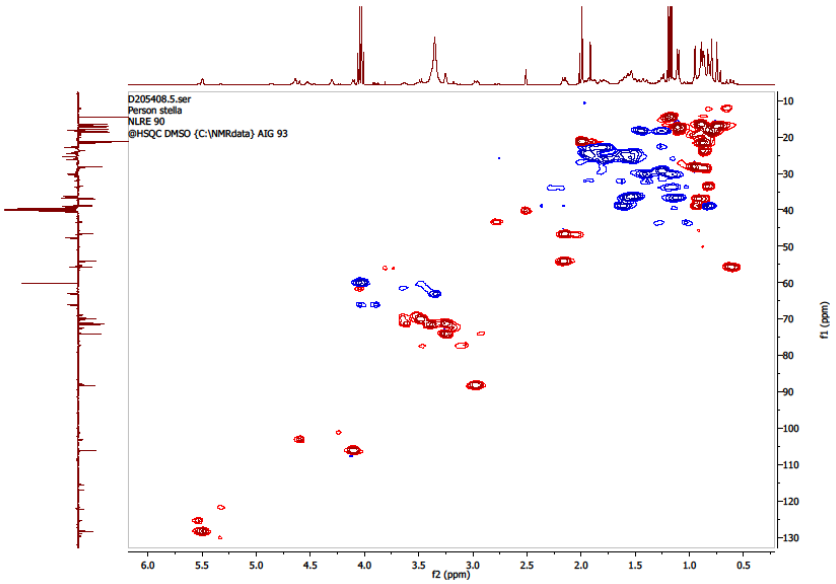

Figure 6: HSQC Spectrum of NLRE 90

3.1.3 Identification of 3, 4-dihydroxybenzoic acid.

NLRS 20 (0.41g) m.p 197^{0}C was isolated as fraction 20 from sephadex column chromatography of the crude methanol extract of *N.latifolia* whole root. The eluting solvent was 100% methanol. It was purified by evaporation of the solvent and crystallization from n-Hex: EtOAc, 50:50. It was a reddish-brown amorphous solid which was sensitive to UV (254nm and 360 nm) giving a brown colour. It gave a dark blue colour when treated with 5% $FeCl_3$ indicating the presence of phenolic hydroxy functions.

Proton and Carbon-13 NMR analyses were carried out in DMSO-d_6. The results are presented in Table 4 and Figure 1c.

Table 4: ^{1}H and ^{13}C NMR spectral data of NLRS 20 in DMSO-d_1, δ (ppm)

Position	^{1}H	^{13}C
1	-	123.28
2	7.34(1H,d)	115.66
3	-	146.18
4	-	151.66
5	7.29(1H,d)	124.03
6	6.79(1H,d)	117.85
C=O	-	170.38

The six carbon signals between 114-151 ppm indicated the presence of aromatic ring. Signal at 170.38 ppm was due to carbonyl function of the carboxylate. Proton NMR (Table 4) indicated 3 protons, 2 coupled to each other attached likely at C-2,C-5 and C-6, oxygenated aromatic quaternary carbon signal at 146.18 and 151.66 ppm, three shielded aromatic methines at 115.66, 124.03 and 117.85 ppm and another quaternary carbon at 123.28 ppm were observed. The 3, 4-dihydroxy benzoic acid structure was suggested by the above spectral information and was confirmed by literature [27, 28].

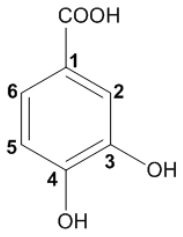

Figure 1c: Structure of 3, 4-dihydroxybenzoic acid

3.2 Anti-Microbial Screening Results

The antimicrobial screening results of strictosamide, quinovic acid and 3,4 - dihydroxybenzoic acid are presented in Tables 5 – 8 and were also discussed.

Table 5: Results of Antibacterial Activities of strictosamide, quinovic acid and 3,4-dihydroxybenzoic acid including gentamicin

Compound	Approximate Concentration mg/ml	Volume used in ml	Average diameter (mm) of zones of inhibition on test organisms										
			E.coli NCTC 10418	*S. aureus NCTC 6571*	*S. typhii LCI*	*Bacillus subtilis L.CI.*	*S. albus LCI*	*S. mutans L.C.I.*	*S. pyogenes L.C.I.*	*P. vulgaris L.C.I.*	*E. aerogenes L.C.I.*	*P. aerugenosa L.C.I.*	*K.pneumoniae L.C.I.*
Strictosamide	200	0.05	17	120	12	12	16	15	15	20	24	10	10
Quinovic acid	200	0.05	14	18	10	12	18	16	17	22	26	12	10
3,4-dihydroxybenzoic acid	200	0.05	18	28	20	10	26	14	14	30	28	16	16
Control: 50% Acetone		0.05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Gentamicin	200	0.05	NA	38	NA	NA	36	30	28	NA	26	NA	NA

Note:

NA - No Action

E.coli (NCTC 10418) - *E. coli* (National Collection of Type Cultures 10418)

S. aureus (NCTC 6571) - *S. aureus* (National Collection of Type Cultures 6571)

L.C.I. - Local Clinical Isolates

Ditch diameter = 6mm

The results of the antibacterial activities of the three compounds shown in Table 5 indicated that they inhibited the growth of all the test organisms (both Gram-positive and Gram – negative). The compounds presented the average diameters of zones of inhibition in the range 30 – 10 mm against all the test organisms. The antimicrobial properties of *Nauclea latifolia* root extracts have been extensively studied because of the ethnopharmacological properties. Ethanolic and aqueous extracts of *N. latifolia* root were very active against four reference strains of the bacteria *S. aureus* ATCC 12600, *B. subtilis* ATCC 6051, *P. aeruginosa* ATCC 10145 and *E. coli* ATCC 11755 in addition to clinical isolates of *S. aureus* and *E. coli*. Combined micro-dilution and agar diffusion methods were used. The ethanolic extract was found to be more active with inhibition zone diameters recorded ranging from 6.60-17mm while MIC varied from 12.50 up to 50mg/ml [4]. Ethanolic (70%) root bark extract was found to decrease the frequency of diarrhea in mice. It also significantly inhibited small intestine motility [29]. These findings confirm the antibacterial and antidiarrheal activities of the root of *N. latifolia* plant.

Alkaloids of which strictosamide is an example have been documented to have antimicrobial (antibacterial and antifungal) activities [30].

The antibacterial properties of quinovic acid have been corroborated by Ryu *et al*., 2010. Pentacyclic triterpenes in general are known to have anti-oxidant, anti-inflammatory, anti-tumor and anti-bacterial effects among others [31].

Terpenoids prevent the formation of ulcers and diminished the severity of existent ulcers [32, 33].

3, 4 – dihydroxybenzoic acid was reported to have activity against *Salmonella thypimurium, Vibro cholera* which not only cause diarrhea but also Salmonellosis with fever and abdominal cramp as additional symptoms. The growth of *E. coli, Listeria monocytogens, S. aureus* and *Bacillus cereus* were also inhibited by the compound [27]. Studies indicated that phenolic compounds – phenolic acids and phenylpropanoid compounds showed important antibacterial activity [34]. Plants rich in tannins and phenolic compounds have been shown to possess antimicrobial activities against a number of micro organisms [30].

Table 6: Results of MIC and MBC in mg/ml of strictosamide, quinovic acid and 3, 4- dihydroxybenzoic acid

Compound		Test organisms										
		E.coli NCTC 10418	***S. aureus NCTC 6571***	***S. typhii LCI***	***Bacillus subtilis L.CI.***	***S. albus LCI***	***S. mutans L.C.I.***	***S. pyogenes L.C.I.***	***P. vulgaris L.C.I.***	***E. aerogenes L.C.I.***	***P. aeruginosa L.C.I.***	***K. pneumoniae L.C.I.***
Strictosamide												
	MIC	25	6.25	50	50	25	25	25	6.25	6.25	50	50
	MBC	50	12.5	100	100	50	50	50	12.5	12.5	100	100
Quinovic acid												
	MIC	50	12.5	50	50	12.5	25	25	6.25	3.125	50	50
	MBC	100	25	100	100	25	50	50	12.5	6.25	100	100
3,4–dihydroxy benzoic acid												
	MIC	25	6.25	12.5	50	12.5	25	25	6.25	6.25	25	25
	MBC	50	12.5	25	100	25	50	50	12.5	12.5	50	50

From Table 6, it could be seen that the MIC values of the compounds were in the range 3.125 – 50mg/ml and MBC values of range 6.25 – 100mg/ml for all the test bacteria. These results showed that the compounds inhibited the growth of the test organisms even at such low concentrations. This has informed the traditional use of the plant to treat ailments caused by these bacteria like diarrhea, dysentery, ulcers, wounds, dental caries, earache, typhoid fever.

Table 7: Results of Antifungal Activities of strictosamide, quinovic acid and 3, 4 - dihydroxybenzoic acid including tioconazole

S/N	Compounds	Approximate Concentration mg/ml	Volume used in ml	Average diameter (mm) of zones of inhibition on test organisms		
				Candida albicans L.C.I	***Aspergillus niger L.C.I.***	***Aspergillus flavus L.C.I.***
1.	Strictosamide	200	0.05	10	12	14
2.	Quinovic acid	200	0.05	12	12	12
3.	3, 4 - dihydroxybenzoic acid	200	0.05	12	10	10
4.	Tioconazole	200	0.05	26	22	24
5.	Control: 50% Acetone		0.05	NA	NA	NA

Note:

NA	-	No Action
E.coli (NCTC 10418)	-	*E. coli* (National Collection of Type Cultures 10418)
S. aureus (NCTC 6571)	-	*S. aureus* (National Collection of Type Cultures 6571)
L.C.I.	-	Local Clinical Isolates
Ditch diameter	=	6mm

The results of the antifungal activities of the compounds showed they had moderate antifungal characteristics as could be seen from Table 7. The average diameters of zones of inhibition ranged from 14 – 10mm. *Aspergillus flavus* was the most susceptible

Table 8: Results of MIC and MFC in mg/ml of strictosamide, quinovic acid and 3,4 – dihydroxybenzoic acid

S/N	Compound		Test organisms		
			Candida albicans L.C.I	***Aspergillus niger L.C.I.***	***Aspergillus flavus L.C.I.***
1.	Strictosamide				
		MIC	50	50	25
		MFC	100	100	50
2.	Quinovic acid				
		MIC	50	50	50
		MFC	100	100	100
3.	3,4–dihydroxy benzoic acid				
		MIC	25	50	50
		MFC	50	100	100

From Table 8, it could be seen that the MIC values of the compounds were in the range 25 – 50 mg/ml and MFC values were of range 50 – 100 mg/ml for all the test organisms. This showed that the compounds had antifungal properties. This has accounted for the ethnomedical use of the plant to treat fungal related ailments like skin diseases, urinary tract infections, asthma, acute pneumonia.

4. Conclusion

The pharmaceuticals: strictosamide, quinovic acid and 3, 4 –dihydroxybenzoic acid were identified through NMR spectroscopy to be present in the whole root of *Nauclea latifolia* plant. These compounds were found to show antimicrobial properties against the test human pathogenic bacteria and fungi from the bioassay results. Their antimicrobial properties compared favourably with the standard antibiotics. They were effective even at low concentrations as were observed from the MIC, MBC and MFC values. The compounds therefore showed broad spectrum antimicrobial effects.

The antibacterial studies showed the efficacy of the compound in the order 3,4 - dihydroxybenzoic acid > quinovic acid > strictosamide. The order of susceptibility of the test bacterial organisms was *E. aerogenes* > *P. vulgaris* > *S. aureus* > *S. albus* > *E. coli* > *S. pyogenes* > *S. mutans* > *S. typhi* > *P. aeruginosa* > *K. pneumoniae* > *B. subtilis.*

The antifungal results indicated the potency of the compounds in the order strictosamide and quinovic acid > 3, 4 - dihydroxybenzoic acid. A. flavus was the most susceptible followed by *A. niger* and *C. albicans.*

In general, the compounds were very effective against the pathogens *E. aerogenes, P. vulgaris, S. aureus, S. albus* and *E. coli*, the causative agents of wound and chest infections, diarrrhea and dysentery, mouth infections, ulcers. The compounds were more effective against the *Aspergillus* species than *C. albicans*. *Aspergillus* species cause asthma, acute pneumonia, abscesses.

These compounds account for the ethnomedical uses of the plants in treating ailments caused by these microbes.

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