Antibacterial Activity of α and β Amyrin Isolated from *Morinda lucida* against Some Multidrug Resistant Enterobacteriaceae

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Abstract

Introduction: Enterobacteriaceae is a family of gram negative bacteria often associated with the gut. They were found to be responsible for a large proportion of serious and life-threatening infections due to the increasing resistance to available antibiotics. In many developing countries different parts of *Morinda lucida* has been used as herbal medicine. The aim of this study was to isolate compound(s) from the most active fraction of M. lucida leaves and to test the activity against some multidrug resistant Enterobacteriaceae.

Methods: *M. lucida* leaves were freshly obtained, shade-dried, grinded and extracted with methanol. This was further fractionated using column chromatography. The different fractions obtained were tested for antibacterial activity against some multidrug resistant members of Enterobacteriaceae. Compound was isolated and elucidated from the most active fraction. This was also tested for antibacterial activity.

Results: The compounds isolated from the *M. lucida* most active fraction are α and β-amyrin mixture. The mixture of α and β-amyrin (0.093 µg/ml) was active against *Klebsiella*, *Pragia*, *Serratia*, *Enterobacter*, *Providencia* species and *E. coli* (zones of inhibition ranged from 15-18 mm).

Conclusion: Implication of the findings in this study presents α and β-amyrin from *M. lucida* as a potential agent for the treatment of some infectious diseases that cannot be treated with commonly used antibiotics. Therefore, this study justifies the use of *M. lucida* in traditional medicine practices and especially as alternative medicine.

Keywords: *Morinda lucida* • α and β-amyrin • Enterobacteriaceae • Multidrug resistance

Introduction

Enterobacteriaceae is a family of bacteria often associated with the gut [1]. Enterobacteriaceae are responsible for a large proportion of serious, life-threatening infections and resistance to multiple antibiotics in these organisms is an increasing global public health problem [2].

Morinda lucida Benth. (Rubiaceae) is a tropical West Africa rainforest tree also called Brimstone tree [3]. The local names of the plant in Yoruba, Igbo and Hausa are Oruwo, Eze-ogu, and Huka respectively [4,5]. In many countries different parts of the plants are used in different ways. The leaves effectively treat and improve all forms of infertility in women. Brimstone tree is locally used in the treatment of irregular menstruation, insomnia and jaundice, also in the treatment of infected wounds, abscesses and chancre [6].

The aim of this study was to isolate a compound from the most active fraction of *M. lucida* leaves and to test its activity against some multidrug resistant Enterobacteriaceae.

Materials and Methods

Collection and identification of plants materials

Morinda lucida leaves were obtained from Ibadan, Nigeria and identified at the Herbarium Section, Department of Botany, Faculty of Life Science, ABU, Zaria and assigned Voucher numbers: 01862 for *M. lucida* (Family: Rubiaceae).

Preparation of plant materials

The *Morinda lucida* leaves specimen freshly obtained were separately washed with water and dried in a shade at room temperature to 1 kg weight. The dried plant samples were then grinded using motar and pestle into coarse powdered form. The powdered samples were stored in an air-tight sterile brown bottle within a desiccator until required for use [7].

Preparation of crude extracts

The extraction and screening was carried out using the method described by Ogbadoyi, et al. and Sankeshwari, et al. [8,9]. One kilograms (1 kg) of the dried powdered samples of *M. lucida* leaves

was extracted with 10 L of methanol for 72 hours. Extracts were filtered using muslin cloth and solvents removed through evaporation using water bath at 40°C. The dried extracts were finally transferred into sterile sample bottles for storage at refrigerated temperature until when required for use [10].

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the extract was determined as described by Adeshina, et al. and Coker and oaikhena with little modification [11,12]. This was carried out for some resistant Enterobacteriaceae isolates at varying concentrations of 200, 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml. Two (2) gram of methanol extract of *M. lucida* leaves was dissolved in 10 ml of sterile distilled water to give the desired concentrations of extract in milligram per millilitre (mg/ml), which were used for the MIC and MBC test. These concentrations in sterile melted Mueller Hinton agar plates were prepared using double dilution method. The solidified leaf extract-agar admixture plates were inoculated with 20 µl of standardized 18 h culture test organism on sterile discs placed on the solidified extract-agar admixture. The inocula were allowed to diffuse into the test agar plates for 30 minutes. The test agar plates were then incubated at 37°C for 18 h and the lowest concentration of the extract in the test agar plates that showed no visible growth was considered as the MIC of the extract against the test organism. An agar plate containing Mueller Hinton agar only was seeded with the test organism to serve as control.

Fractionation of the extract and elucidation of chemical constituents

Thin Layer Chromatography (TLC) of plant extracts: TLC of the plant extract was carried out. Different solvent systems based on their polarity were used. Two (2) µl of plant extract was applied to the TLC chromatogram. Solvent systems used are Hexane and Ethyl Acetate, 9:1, 8:2, 7:3, etc. Individual Rf for each spot was measured. TLC spots were visualized under UV light and adequate TLC reagents were used.

Fractionation was carried out using column chromatography with the best solvent system from the TLC profiling. Chemical elucidation was carried out using FTIR, NMR (H, ^{13}C, and 2D).

Column chromatography: The column chromatographic method as described by Ewansiha, et al. with little modification was used to separate the fractions of the methanol extracts [13]. The column was prepared by plugging a Pasteur pipette with a small amount of cotton using a simple dry-pack method and with a wood applicator stick it was tamped down lightly. One hundred milligram silica gel was added as the stationary phase. The column was next pre-eluted by the addition of solvent (hexane) and it was allowed to flow slowly down the column by gravity. The column was loaded with the sample by the dry method which involves dissolving 5 g of the extract mixed with silica gel in a solvent (methanol), allowed to air-dried, crushed with sterile pestle and in a crucible before addition. It was then eluted as necessary by introducing more solvent (starting from the less polar solvent hexane 100%, hexane/ethyl acetate; ethyl acetate 100%; ethyl acetate/methanol to more polar solvent methanol) through the column which also prevents the silica gel from going dry. The fractions were collected in bottles according to their colour development.

Thin Layer Chromatography (TLC) of the pulled fractions: The different fractions obtained were pulled together based on their TLC profile (fingerprint). Thin layer chromatography was used, in which the TLC plate was spotted with the different fractions obtained and developed with different solvent systems in a chromatographic tank.

Antimicrobial analysis of *M. lucida* fractions and the isolated compound: Antibacterial susceptibility was tested using the modified Agar well diffusion method as used by Adeshina, et al. and Coker and Oaikhena. The surface of sterile Mueller Hinton agar was flooded with 1 ml of 0.5 Mc Farland standardized inoculum and allowed to cover the entire surface, after which the excess was discarded into a container of disinfectant. The inoculated Mueller Hinton agar surface was allowed to dry, after which wells were bored on the surface of the inoculated agar plates using sterile stainless cork borer (10 mm).

After adding a drop each of molten Mueller Hinton agar to seal the bottom of the wells, 20 µl of the different fractions were transferred into the wells using micro pipette. The wells were sufficiently spaced to prevent the resulting zones of inhibition from overlapping. Pre-incubation diffusion time (45 minutes) was allowed, after which the petri-dishes were incubated at 37°C for 24 hrs.

The zones of inhibition surrounding the wells were measured in millimeters using a ruler [14]. Clear inhibition zones around the wells indicated the presence of antimicrobial activity. The mean of the resulting zones of inhibition were determined.

Results

Fractionation test result

The MIC of the *M. lucida* active extract against the tested bacteria was within the range of 25 to 100 mg/ml (Table 1). One hundred and twenty-six (126) fractions were obtained and were pulled together to 27 fractions (M1-M27) based on their TLC fingerprint profiles (Table 2). Fraction M25 showed better activity with a zone of inhibition of 15.3 mm, compared to the other fractions. Others following are M18 and M24 having 15.1 and 15.0 mm respectively (Table 3).

Table 1. Minimum inhibitory concentrations of M. lucida against the isolates.

Isolate code	Organism	MIC (mg/ml) *M. lucida*
228	*Escherichia coli*	100
2648	*Escherichia coli*	100
154	*Klebsiella oxytoca*	25
182	*Salmonella gallinarum*	100
210	*Salmonella Arizonae*	100
184	*Serratia marcescens*	100
2602	*Serratia marcescens*	100
2638	*Enterobacter gergoviae*	25

209	*Pantoea agglomerans*	100
190	*Providencia rustigianni*	100
2557	*Budvicia aquatic*	100
7617	*Pragia fontium*	50

Table 2. *M. lucida* pulled fractions.

S. no.	Pulled fractions codes	Fractions	Number of spots
1	M1	1-7	4
2	M2	8-9	5
3	M3	10-16	6
4	M4	17-18	6
5	M5	19-21	7
6	M6	22-23	8
7	M7	24-26	7
8	M8	27-29	6
9	M9	30-34	5
10	M10	35	5
11	M11	36	5
12	M12	37	4
13	M13	38-41	6
14	M14	42-44	6
15	M15	45-55	4
16	M16	56-61	3
17	M17	62	3
18	M18	63	4
19	M19	64-65	4
20	M20	66-67	5
21	M21	68-76	4
22	M22	77	3
23	M23	78-79	3
24	M24	80-106	3
25	M25	107-113	2 (tailing)
26	M26	114-120	2 (tailing)
27	M27	121-126	2

Table 3. Zone of inhibition (mm) for the *M. lucida* pulled fractions I.

S. no.	Isolate code	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21	M22	M23	M24	M25	M26	M27
1	228	10	10	10	14	16	17	12	11	14	16	20	18	19	14	10	12	16	18	13	15	14	14	13	15	17	16	18
2	2648	10	10	11	15	13	13	11	14	24	12	18	19	13	12	10	10	10	10	10	11	10	10	10	13	10	10	10
3	154	10	10	11	13	11	10	10	10	10	10	18	19	10	10	20	16	15	20	13	10	12	12	16	24	25	21	22
4	2603	10	10	14	12	11	10	10	10	15	12	14	16	10	10	10	10	16	18	21	14	19	10	14	26	18	10	11
5	169	10	10	12	11	14	11	14	10	10	10	12	15	11	10	20	19	21	22	20	20	11	12	14	21	27	18	15
6	210	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	11	11	10	10	10	10	10	10	10
7	226	10	10	15	14	13	16	20	15	10	13	12	10	11	11	12	12	13	14	15	25	13	13	16	15	16	14	14
8	182	10	10	10	12	13	14	10	10	10	10	10	10	10	10	11	11	14	15	13	15	13	14	10	13	12	10	10
9	1631a	10	10	10	12	10	10	12	15	12	10	13	14	10	12	10	13	12	15	18	10	11	10	14	13	18	14	10
10	184	10	10	10	10	16	18	12	10	15	12	12	10	12	11	14	16	20	22	17	15	20	14	13	14	19	15	16
11	2602	10	10	11	14	13	10	10	10	10	10	11	13	15	12	10	18	14	16	13	11	25	11	15	16	17	18	11
12	2638	10	10	10	13	12	14	10	10	10	10	10	10	10	10	13	10	12	16	16	10	19	10	11	12	10	10	10
13	209	10	11	12	13	10	10	10	10	11	10	11	11	10	11	10	11	10	10	10	10	10	10	10	10	10	11	10
14	190	10	10	10	12	11	11	11	10	10	10	15	12	10	10	10	10	13	14	14	12	12	10	12	17	16	20	13
15	2557	10	10	10	13	11	11	11	10	10	10	10	12	10	10	10	10	10	10	10	10	10	10	10	10	10	11	10
16	7617	10	10	10	12	10	10	10	10	10	10	10	10	10	10	10	11	12	11	14	10	11	11	11	11	10	10	10
Average		10	10.063	11	12.5	12.13	12.188	11.438	10.938	11.938	10.938	12.875	13.063	11.313	10.813	11.875	12.438	13.625	15.063	14.25	13.063	13.75	11.313	12.438	15	15.313*	13.625	12.5

Key: 228, 2648= *E. coli*; 154, 2603, 169= *Klebsiella* species; 210, 226, 182, 1631a=*Salmonella* species; 184, 2602=*Serratia* species; 2638= *Enterobacter* species; 209= *Pantoea agglomerans*; 190= *Providencia* species; 2557= *Budvicia* species and 7617= *Pragia fontium*.

Figure 1A-C showed the Thin Layer Chromatography (TLC) profile of some of the pulled fractions. From the TLC, the isolated compound had an Rf value of 0.74 and it is pink in color after spraying and heating (colourless under UV) (Figure 1D). The compound was whitish crystal and completely soluble in chloroform.

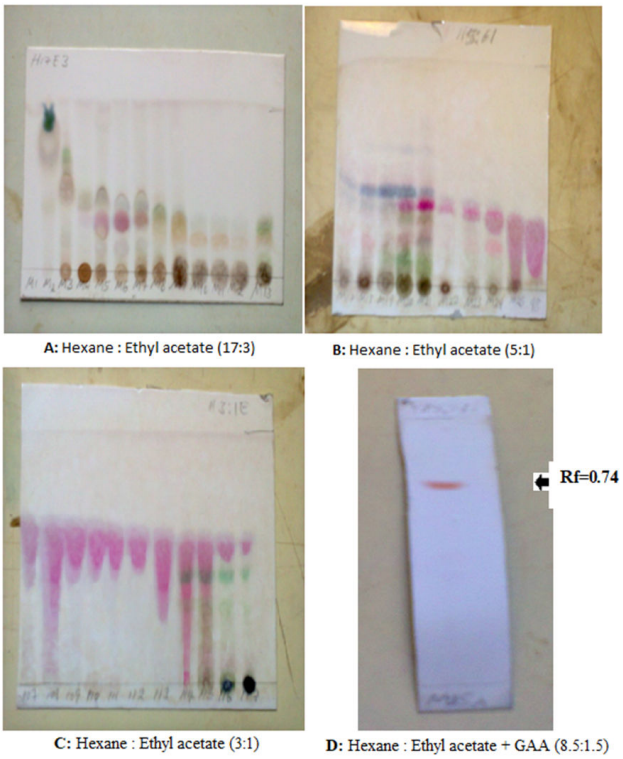

Figure 1. TLC profile of some of the pulled fractions (A, B and C) and that of the isolated compound (D).

Compound elucidation

Figures 2-8 showed the FTIR spetrum, and 2D NMR (Proton, Carbon 13, HSQC, HMBC, COSY and DEPT) spectra of the isolated compound. The peaks observed at 3414.2, 2926.0, 2855.1 and 1688.5 are functional groups indications of Hydroxyl (OH), asymmetric Methyl (-CH_3) stretch, symmetric Methyl (-CH_3) stretch and Alkenyl (C=C) stretch respectively in the compound isolated (FTIR spectrum). The proton spectrum of M25A in CC13 (400 Hz) showed the presence of eight methyl singlets, two close and similar triplets for two carbinylic protons at δ_H 5.27 (t, J=3.2, H-12 for α-amyrin) and 5.24 (t, J=3.5, H-12 for β-amyrin). An oxymethine proton (H-3) at δ_H 3.22 (α-amyrin) and 3.20 (β-amyrin) was also observed and integrated for two protons approximately. The rest of the signals were for methyl and methylene as well as other methine protons but they were overlapped for the two compounds. The ^{13}C spectrum showed the distinct carbon atoms for the compounds while the rest of the carbons were close together or overlapped. For alpha amyrin the C-12 signal was observed at δ_C 123.03 and the C-13 signal at 139.7 ppm while for beta amyrin, the C-12 signal was observed at 120.91 and the C-13 at 143.43 ppm. The oxygenated C-3 for alpha amyrin was at 79.99 and for beta amyrin at 79.20 ppm. Based on the chemical shifts reported for the compounds and compared to our experimental values (Table 4), M25A was identified as a mixture of α and β-amyrins (Figure 9).

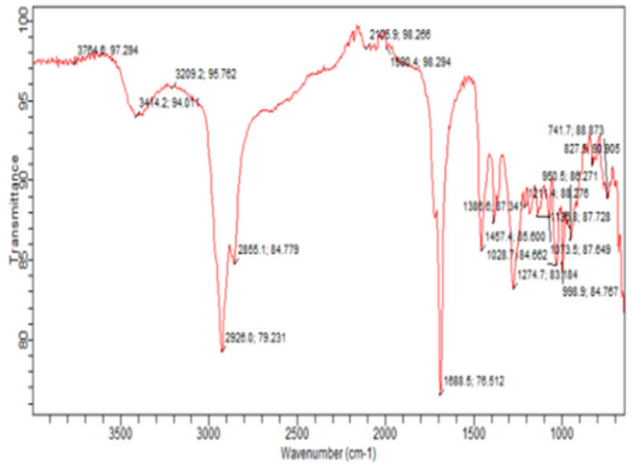

Figure 2. M25A FTIR spectrum.

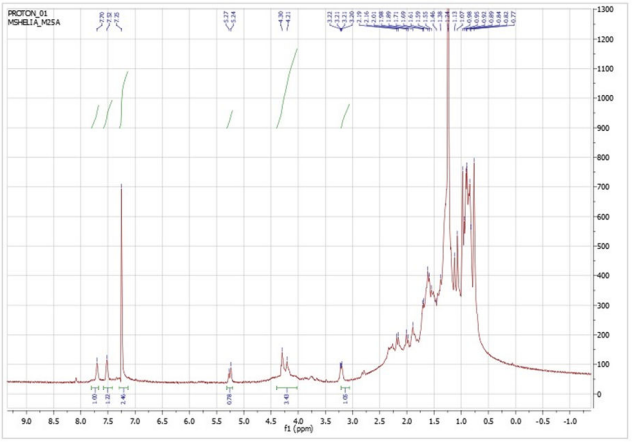

Figure 3. M25A NMR_Proton spectrum.

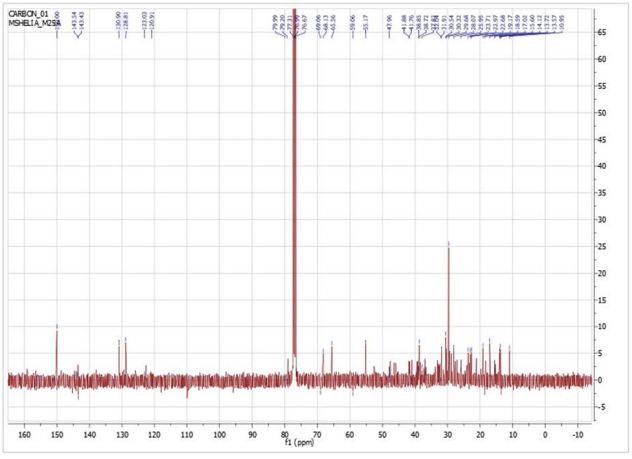

Figure 4. M25A NMR_Carbon13 spectrum.

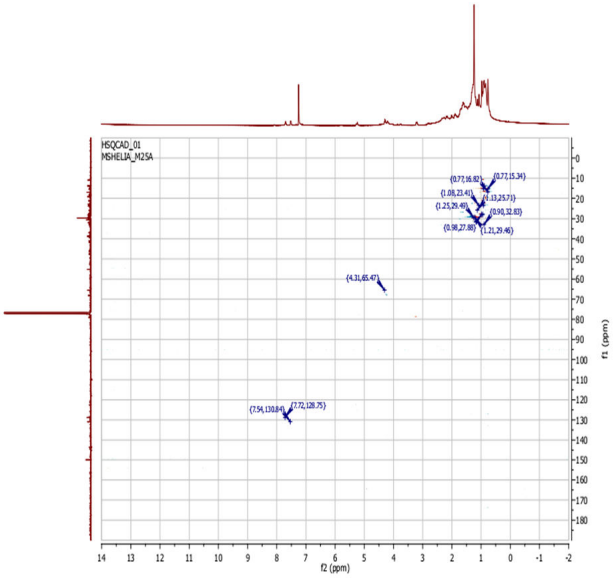

Figure 5. M25A NMR_HSQC spectrum.

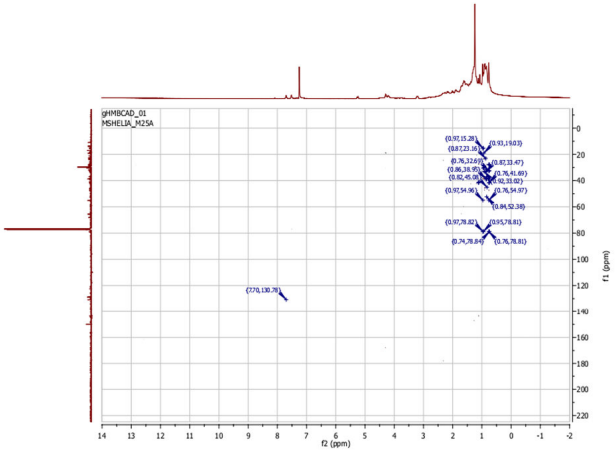

Figure 6. M25A NMR_HMBC spectrum.

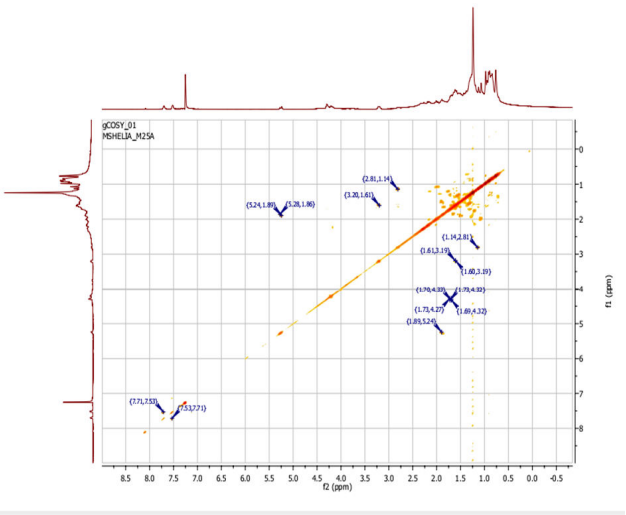

Figure 7. M25A NMR_COSY spectrum.

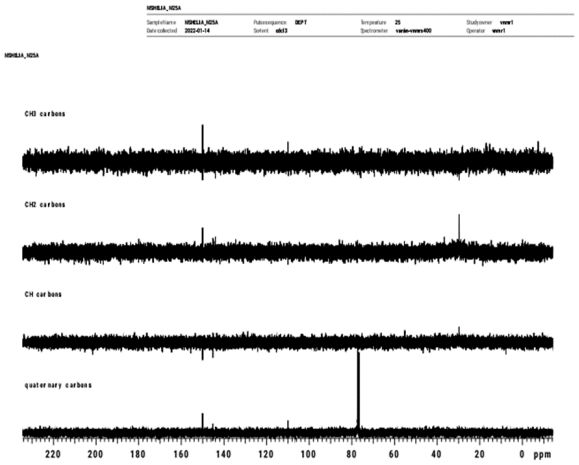

Figure 8. M25A NMR_DEPT spectrum.

Table 4. Characterization of the isolated compound M25A.

Position	^{1}HNMR	^{1}HNMR α-Amyrin Lit.	^{1}HNMR β-Amyrin Lit.	^{13}C-NMR	^{13}C-NMR α-Amyrin Lit.	^{13}C-NMR β-Amyrin Lit.
1	1.55, 1.46	-	1.55 (Hb-1) 1.49 (Ha-1)	38.72	38.6	38.79
2	1.55, 1.59	1.61	1.52 (Hb-2) 1.55 (Ha-2)	23.71	23.8	27.44
3	4.3 3.20 dd (4.0, 8.0)	4.48 dd	3.20 dd (4.4,11.5)	79.25	81.18	79.24
4	-	-	-	37.82	37.9	38.99
5	0.82	0.81	0.71	55.17	55.46	55.37
6	1.46, 1.38	1.51, 1.34	1.53 (Hb-6), 1.30 (Ha-6)	18.59	18.45	18.58
7	-	-	-	31.91	33.09	32.85
8	-	-	-	41.52	40.02	40.21

9	1.71	1.54	1.95	47.96	47.84	47.43
10	-	-	-	36.76	36.99	37.15
11	1.89	1.89	1.84	22.97	23.6	23.75
12	5.24	5.10 t (3.6)	5.16 t (3.5)	123.03, 120.91	124.5	121.93
13	-	-	-	143.43	139.8	145.41
14	-	-	-	41.88	42.4	41.92
15	-	-	-	28.07	28.29	26.36
16	-	-	-	25.95	26.8	27.14
17	-	-	-	32.04	33.95	32.7
18	1.24, 1.89	1.29	1.89	59.06	59.26	47.84
19	1.38, 1.59	1.38	1.59	39.55	39.81	47.03
20	1.98	1.98	-	39.55	39.84	31.3
21	1.69	-	1.66	30.54	31.47	37.35
22	-	-	-	41.76	41.7	34.94
23	0.84	0.85 s	0.77 s	28.07	28.27	15.71
24	0.84, 0.98	0.84 s	0.98 s	15.6	16.96	28.31
25	0.95, 0.92	0.96 s	0.92 s	14.12	15.96	15.8
26	0.98, 0.95	0.98 s	0.94 s	17.02	17.71	17.01
27	1.07, 1.13	1.04 s	1.11 s	22.97	23.43	26.21
28	0.77	0.78 s	0.81 s	29.68	29.06	28.62
29	0.77	0.77 d	0.85 s	17.02	17.02	33.56
30	0.82	0.83 d	0.85 s	22.68	21.64	23.91

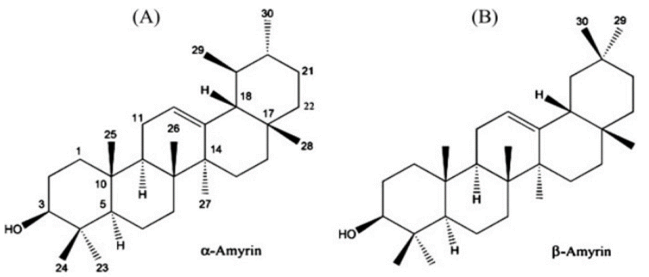

Figure 9. Structure of the proposed isolated compounds (Alpha and Beta amyrin).

The isolated compound (alpha and beta amyrin) (0.0928 µg/ml) showed better activity on *Klebsiella*, *Pragia*, *Serratia*, *Enterobacter*, *Providencia*, species and *E. coli* with zones in the range of 15-18 mm (Table 5). The zones of inhibition of the isolated compound was compared with that of some antibiotics, whereby alpha and beta amyrin had better activity (Figure 10).

Table 5. Zone of inhibition (mm) for the isolated compound (M25A).

S. no.	Isolate code	Organism	Zone of inhibition
1	228	*E. coli*	15*
2	2648	*E. coli*	14
3	154	*Klebsiella* spp.	14

4	2603	*Klebsiella* spp.	18*
5	169	*Klebsiella* spp.	13
6	210	*Salmonella* spp.	13
7	226	*Salmonella* spp.	13
8	182	*Salmonella* spp.	14
9	1631a	*Salmonella* spp.	14
10	184	*Serratia* spp.	15*
11	2602	*Serratia* spp.	17*
12	2638	*Enterobacter* spp.	15*
13	209	*Pantoea* spp.	14
14	190	*Providencia* spp.	15*
15	2557	*Budvicia* spp.	14
16	7617	*Pragia* spp.	18*
Average			14.75

Key: *= Better activity

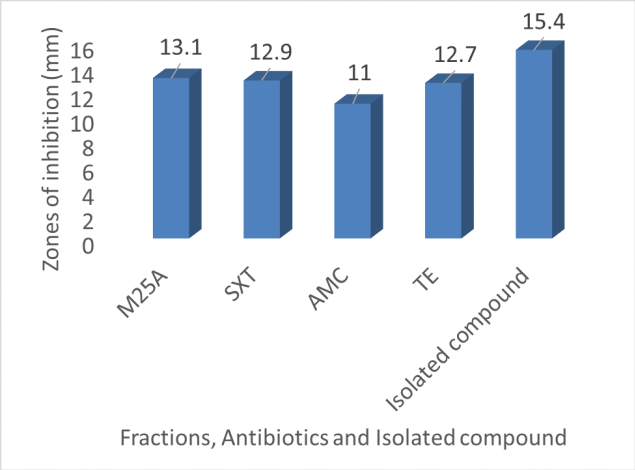

Figure 10. Average zone of inhibition (mm) for the *M. lucida* methanol extract most active of the pulled fractions and the isolated compound.

Discussion

The proton spectrum for the mixture in CDCl3 showed two close and similar triplets for two olefinic protons at δ_H 5.28 (t, J=3.5, H-12 for α-amyrin) and 5.25 (t, J=3.5, H-12 for β-amyrin). This is characteristic for triterpenes having a C-12 olefinic proton such as amyrins and other similar triterpenes such as oleananes, ursanes and amyrins having a C=C double bond between C-12 and C-13. The triplet multiplicity is due to coupling with the H-11 protons usually at 1.84 and 1.87 ppm. An oxymethine proton (H-3) at δ_H 3.23 (α-amyrin) and 3.20 (β-amyrin) was also observed and integrated for two protons approximately. The rest of the signals were for methyl and methylene as well as other methine protons but they were overlapped for the two compounds. The ^{13}C spectrum showed the distinct carbon atoms for the compounds while the rest of the carbons were close together or overlapped. For alpha amyrin the C-12 signal was observed at δ_C 122.8 and the C-13 signal at 139.7 ppm while for beta amyrin, the C-12 signal was observed at 125.8 and the C-13 at 145.2 ppm. The oxygenated C-3 for alpha amyrin was at 79.17 and for beta amyrin at 79.21 ppm. The rest of the carbon signals were in good agreement with literature reports. Based on the chemical shifts reported for the compounds and compared to our experimental values (Table 5), the fraction was identified as a mixture of α and β-amyrins [15,16].

The isolated compound (alpha and Beta amyrin) in M. lucida methanol extract is from the triterpenes family. It is a pentacyclic triterpenoid [17]. The isolated compound (0.0928 µg/ml) showed better activity against *Klebsiella* spp., *Pragia* spp., *Serratia* spp., *Enterobacter* spp., *Providencia* species and *E. coli*. Diaz-Ruiz, et al. reported that α-amyrin and β-amyrin isolated from copal (*Bursera* spp) and *B. crassifolia* respectively inhibited the growth of some bacteria, including *S. mutans*, at concentrations ranging from 64 to 1088 µg/ml. β-Amyrin acetate isolated from *Heliotropium marifolum* showed potent activity against *Penicillum chrysogenum*, *Escherichia coli* and *Klebsiella pneumoniae* [18]. Johann, et al. reported that Amyrin formate and acetate derivatives were the most active compounds, inhibiting all the opportunistic *Candida* species when tested in concentrations from 30 to 250 mg/ml [19]. Other studies have demonstrated that the α/β-amyrin mixture also has anti-inflammatory, gastroprotective, antiallergenic and antinociceptive activities [20].

Conclusion

Implication of the findings in this study presents α and β-amyrin from *M. lucida* as a potential agent for the treatment of some infectious diseases that cannot be treated with commonly used antibiotics. Therefore, this study justifies the use of M. lucida in traditional medicine practices and especially as alternative medicine.

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